STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

VOLUME 17

1942

Published by
BIOTECH PUBLICATIONS
GENEVA, N. Y., U. S. A.

Printed in the U. S. A.

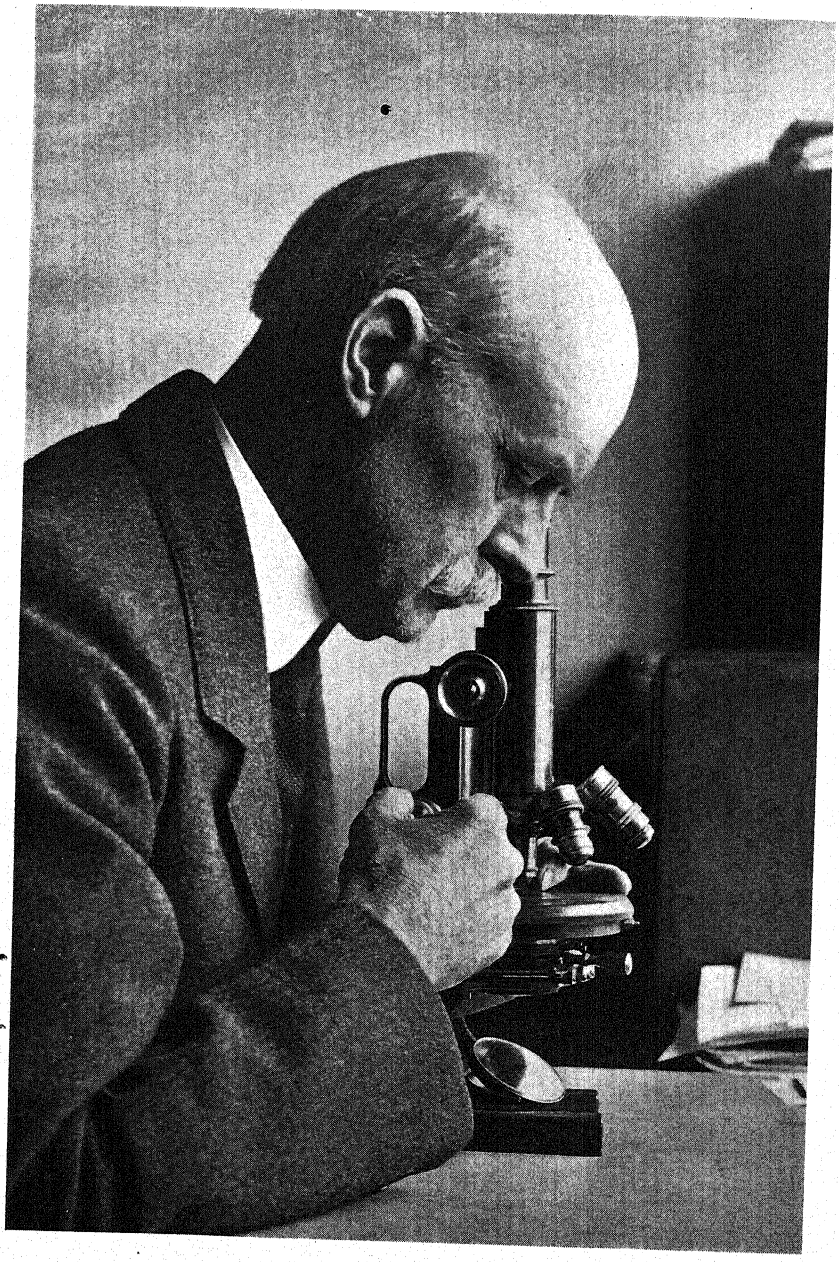
CONTENTS

No. 1, January 1942

No. 2, April 1942

No. 3, July 1942

No. 4, October 1942



DR. FRANK B. MALLORY
1862–1941

STAIN TECHNOLOGY

VOLUME 17 JANUARY, 1942 NUMBER 1

FRANK B. MALLORY

In Memoriam

Dr. Frank B. Mallory, long a member of the Executive Committee of the Stain Commission, died on Sept. 27th, 1941, at the age of 78 years. His passing deprives us of one of the earliest and most enthusiastic of the collaborators in the standardization of stains in America.

Dr. Mallory was associated for about fifty years with Harvard Medical School and Boston City Hospital. He was taken on the staff of Harvard Medical School in 1890 as Assistant in Histology and became Assistant in Pathology the following year. From then on he was a member of the latter department, becoming Professor in Pathology in 1928, and Professor Emeritus in 1932. His connection with the Boston City Hospital began in 1891 when he was made Assistant to the Pathologist; from 1908 till 1932 he held the position of Pathologist. His retirement in 1932, when he reached the age limit of 70, was hardly more than nominal; altho his title after that was merely Consulting Pathologist, he continued to come to the laboratory regularly until about six months before he died. He maintained active interest in the work of the department up to the very end, altho much of his last six months was spent in Florida.

His interests were by no means confined to the work of his laboratory, as he had various connections with scientific societies, and was especially active in affairs of the Association of Pathologists and Bacteriologists. He became treasurer of this Association in 1911 and remained in that office until 1940. In 1925 he took over the editorship of the official organ of this Association, the American Journal of Pathology, which was at that time in such poor shape financially that building it up seemed a very appropriate interest of the society's treasurer. In a few years he put the journal on its feet financially, increased its size and assured greater promptness of publication than it had known before. He remained its editor until 1940.

In the field of technic he became particularly well-known, following the appearance of the book, Pathological Technique, which he

wrote in collaboration with Dr. J. H. Wright; the first edition appeared in 1897, and the book ran thru eight editions, the last dated 1924. Later, after Dr. Wright's death, it seemed best to prepare a new book to take the place of this; as a result, in 1938, a book of the same title, but nevertheless a distinctly new contribution, under Dr. Mallory's name alone, was published. During this period he was quite generally recognized as a leading authority on the subject covered by this publication.

As a result of his pre-eminence in this field, he was one of the five men appointed in 1921 by the Chairman of the Division on Biology and Agriculture of the National Research Council to form a Committee on the Standardization of Biological Stains. When the Commission on Standardization of Biological Stains was organized a year later he became the representative on it of the Association of Pathologists and Bacteriologists and thus a member of its Executive Committee. He remained on the Executive Committee until 1939, when he felt it necessary to retire because of his age; at that time there were only two other members of the original committee still active on it. Dr. Mallory remained a member of the Commission till his death, and his interest in the work continued. Often during the last ten or twelve years of his membership on the Executive Committee he apologized for not being more active on it; but altho it is true that pressure of other duties did keep him from being as active in this work as he might have wished, his enthusiasm for it was a constant source of inspiration whenever personal contact with him was possible. And, from the comments of his associates, it is gathered that this same enthusiasm was responsible for his success in the various activities with which he was more intimately associated.

PROGRESS IN THE STANDARDIZATION OF STAINS

Co-operation among the Americas

Prior to the first World War, all America was dependent upon Germany for biological stains. This source of supply was cut off early in the war; but because of existing stocks in the hands of dealers, as well as of laboratories where the stains were used, a shortage was prevented for two or three years. By 1917, however, when the United States entered the war, these stocks were getting exhausted; and the increased demand, due to the equipment of new Army and Navy laboratories, soon caused the shortage to become acute in North Americа. It is not certain what the situation was in South America at that time, but it is clear that the demand for biological stains must have been very much smaller than in the United States and Canada. The demand for biological stains was met in the United States after a fashion by the newly created dye industry. This industry was operating under difficulties at the time, and their products were not always reliable, even for the much cruder use to which dyes are put in the textile industry. Naturally, they were not very reliable when employed as biological stains.

No steps were taken during the war to make America permanently independent of Germany in the matter of biological stains; but shortly after the war had ended, when it became evident that the embargo on dyes was not likely to be lifted for some time, the Biological Stain Commission was formed with the original intention of learning which American sources of stains were reliable. The scope of the Commission has since extended much beyond the original intention, but that does not alter the fact that it has accomplished what it originally set out to undertake. Reliable biological stains can now be obtained in America.

There seems little question but that the situation faced in North America during the first World War must now exist in the South American countries. The last 20 years have seen such a rapid development of scientific work in the Southern part of this hemisphere that the demand for biological stains in those countries must approximate what it was in North America three decades ago. Also, just as North America was then using German stains exclusively, there are natural reasons for assuming that this was the primary source of South American stains at the outbreak of the present war. Now that this source has been cut off by the War, it would not be at all surprising if some of the laboratories in the American countries to the south of us might be having difficulty in getting satisfactory stains.

It is much to be hoped that the United States can be of some assist-

ance to the other American countries in locating reliable sources of these products. Steps are being taken at present to contact users of stains thruout Pan America and to learn what their problems may be.

Fortunately, in this connection, a member of the Stain Commission and former histologist in the Commission laboratory, is soon to be sent to South America as a special representative of one of the largest optical companies in the United States. The member in question is Dr. J. A. DeTomasi, who was at Geneva for three or four years, and has been, since that time, on the editorial board of Stain Technology. Altho now in the commercial field, his interest in the work of the Stain Commission still continues. We know it is his firm intention to see that knowledge of the work of the Stain Commission is spread among those with whom he comes into contact, and his work is likely to take him into biological laboratories in various countries.

In addition to letting our colleagues in the other Americas know that stains are available in the United States market, each batch of which has been tested and approved by workers skilled in the necessary technical methods, there are, undoubtedly, other ways in which coöperation can be effected. At the present time, membership in the Stain Commission is confined mostly to the United States, altho there are a few members in Canada and one or two in the southern countries. This has come about purely because the American dye industry started in the United States and the acute need of standardizing the products became evident in this country more quickly than elsewhere. There must, however, be many laboratory workers in South and Central America at present whose coöperation would be well worth while. In the hope of securing the broader point of view which contact with the scientists of other countries will give us, special efforts are now being made to approach such men and to secure their coöperation. Some of them may well be valuable material for membership on the Stain Commission. Theoretically the members of the Commission are individuals who are able and willing to assist in testing stains submitted for certification; and it is obvious that such assistance could not be given in any practical way by members as distant as South America. On the other hand, recent years have seen so much more of the actual work of testing stains carried on in the Commission laboratory, by Commission *employees*, that *members* are now rarely called on for the purpose. That being the case, distantly located members are not out of the question. Undoubtedly it will be to the advantage of the Stain Commission to take on new members from these other countries; and at the same time these new members would profit by learning from us how the staining problems have been solved which have faced workers in the United States during the last 20 years.—H. J. Conn.

THE USE OF DARKFIELD ILLUMINATION FOR THE STUDY OF STAINED BLOOD FILMS[1]

P. H. RALPH, *Department of Zoology, University of Michigan, Ann Arbor, Mich.*

ABSTRACT.—It is shown that by the proper use of a modern darkfield condenser, it is possible to eliminate the difficulties ordinarily encountered in the examination of stained blood smears under darkfield illumination. For hematological studies the proposed method has the following advantages over the usual brightfield procedure: structures not otherwise seen become visible and resolvable; the greater range of size, refractivity, and color of structures furnishes criteria for a more detailed differentiation of cell types.

Much has been written concerning the appearance of blood cells under darkfield illumination. Aynaud (1913), Stübel (1914), Malowist (1910), Brugsch and Schilling (1908), Gaidukov (1910) and others have found that the use of darkfield makes possible a more critical resolution of small structures in living blood than any other method. Michaelis (1905) and Cesaris Demel (1913) first examined stained blood smears by this method. Due to diffraction rings, the use of imperfect equipment, technical difficulties, etc., the study of stained blood cells by this method never came into common use. In the course of an extensive study of amphibian blood we have found that proper use of the modern darkfield condenser eliminates these difficulties.

The equipment used in the present study was a Spencer binocular microscope with 10× achromatic eyepieces and a fluorite oil immersion N. A. 1.3, 97×, and focal distance 1.8 mm. For brightfield observations a Spencer 1.25 Abbé condenser was used and for darkfield work a Spencer bispheric darkfield condenser. A 6-volt, 108-watt lamp with a focusing condenser served for both bright and darkfield work. A blue ground glass was placed over the light source for brightfield observations. Complete directions for use of the darkfield condenser are given by Gage (1920).

For purpose of comparison of the two methods of illumination and description of the differences between them reproductions of two photographs are included, one taken with brightfield and the other with darkfield illumination. The color differences will be described

[1]Contribution from the Zoological laboratories of the University of Michigan.

in the text. The cells are from a smear of Guinea pig bone marrow stained with Wright's stain. A Zeiss Phoku camera and Eastman Super XX Panchromatic film were used for photographs. Color photographs may be made with the same apparatus and Eastman indoor (type A) Kodachrome film and have been employed in connection with this study. It has been found desirable when studying microscopic objects in which color is used as a criterion to name the colors by comparing them with an accepted color standard and nomenclature. The color terminology is that given by Ridgeway (1912). In some cases descriptive color names are given in parenthesis.

Figure 1 shows a megakaryocyte (center) and two granular leucocytes (upper right and left) photographed in brightfield.

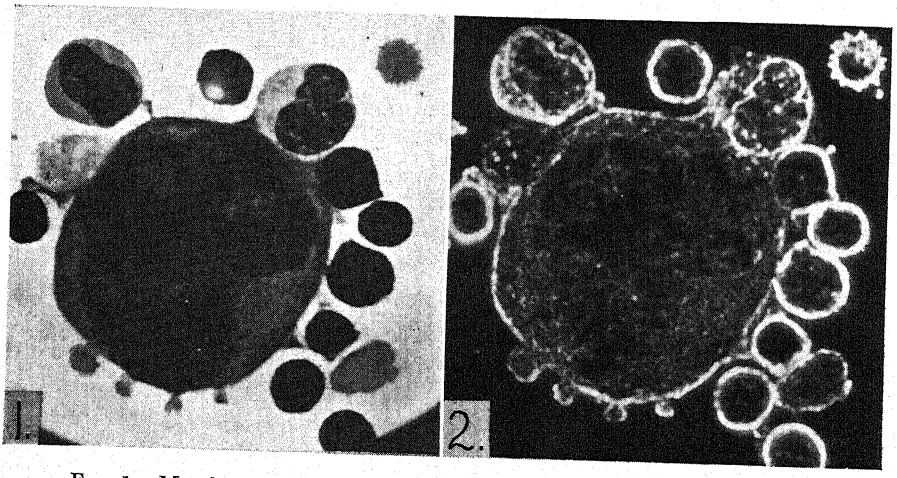


FIG. 1. Megakaryocyte and cells of Guinea pig bone marrow in lightfield.
FIG. 2. Megakaryocyte and cells of Guinea pig bone marrow in darkfield.

Figure 2 shows the same cells in darkfield. In brightfield the nucleus of the megakaryocyte was colored between an ultramarine blue (true blue) and a roslyn blue (dark bluish violet). The cytoplasm about the edge of the cell and the cytoplasmic protrusions were colored from a bradley's blue (blue-violet) to a light violet blue. No distinct granules could be seen. The lavender violet cytoplasm at the indentation of the nucleus was finely mottled but no discrete granules could be observed. The granulocytes at the upper right and left contained a few tyrian pink (light violet-red) granules but different types could not be discerned.

Under darkfield the inter-chromatin spaces of the nucleus of the megakaryocyte were colored a tyrian pink. The chromatin was

not colored. The cytoplasmic membrane was highly refractive and colored from grenadine red (red-orange) to cadmium yellow (orange-yellow). The cytoplasm itself was filled with tiny granules ranging in color from vivid green (bluish green) to viridine green (yellowish green). The color of the granules at the edge of the cell was masked by the basophilia of the cytoplasm. The granules here were colored a grenadine red, and were very dully refractive. In the granulocyte at the upper right the cytoplasmic membrane was not visible, and the granules were colored from venice green (light greenish blue) to a neuvider green (light bluish green). In the one to the upper left the cell membrane was quite visible and in color was a light chalcedony yellow (light greenish yellow). The greater number of the granules were colored from a neuvider green to a clear yellow green. In addition six granules were present which were a light rosolane purple (reddish violet). In an extensive study of frog blood it was found that the color, size, and refractivity of the granules was constant and characteristic for each cell type. It was found that darkfield may also be used for stained sections and intravitam stained cells of all types.

Two chief advantages of darkfield illumination for hematological studies may therefore be mentioned: first that structures, not seen in brightfield, are rendered visible and resolvable, and second, that the greater range of size, refractivity, and color of structures furnishes criteria for a much more detailed differentiation of cell types. The first is due to the fact that a deflection of light rays, because of a difference in refractive index, is all that is necessary to produce an image, while in brightfield the object must be sufficiently large to stop or modify the light rays. The wider variation in color is due to the fluorescent activity of the combined stain and structure which alters the wave length of the light deflected from its surface. The present communication is designed to re-emphasize the value of the darkfield in the study of stained microscopic objects and the use of a standard nomenclature to extend the accuracy of description.

REFERENCES

AYNAUD, M., and JEANTET, P. 1913. Applications de l'ultramicroscope en hématologie. Traité du sang. Gilbert et Weinberg, Paris.

BRUGSCH, TH., and SCHILLING, V. 1908. Die Kernform der lebenden neutrophilien Leukozyten beim Menschen. (Beobachtungen im Dunkelfeld). Fol. Hematol., **6**, 327–36.

CESARIS DEMEL, A. 1913. Nuove osservazioni di ultramicroscopia sul sangue. Pathologica, **6**, 587–96.

GAGE, S. H. 1920. Modern dark-field microscopy and the history of its development. Trans. Amer. Micr. Soc., **39**, 95–141.

GAIDUKOV, N. 1910. Dunkelfeldbeleuchtung und Ultramikroskopie in der Biologie und in der Medizin. D. G. Fischer, Jena.

MICHAELIS, L. 1905. Ultramikroskopische Untersuchungen. Virchow's Archiv., **179**, 195–201.

MALOWIST, S. 1910. Ultramikroskopische Untersuchungen des Blutes bei Gesunden und Kranken. Emil Lehman, Leipzig.

RIDGEWAY, R. 1912. Color standards and nomenclature. Washington, D. C.

STÜBEL, H. 1914. Ultramikroskopische Studien über Blutgerrinnung und Thrombocyten. Pflüg. Arch. für Physiol., **156**, 361–400.

MECHANISM OF THE SELECTIVE ACTION OF EOSIN-METHYLENE-BLUE AGAR ON THE ENTERIC GROUP

E. S. WYNNE, L. J. RODE, AND A. E. HAYWARD, *Department of Botany and Bacteriology, University of Texas, Austin, Tex.*

ABSTRACT.—The actual mechanism of the differentiation of lactose-fermenting and non-lactose-fermenting organisms on eosin-methylene-blue medium is not reported in the literature. The present study is an attempt to elucidate this problem.

The color of colon forms on E.M.B. agar was found to depend on two factors: (1) the reaction of eosin with methylene blue to form a dye compound of either acidic or neutral nature, and (2) the production, by lactose-fermenting colonies, of a sufficiently low pH so that this dye compound is taken up by individual cells of the colony. Non-lactose-fermenting organisms are not colored because the compound is not taken up in alkaline reaction.

An explanation is offered to account for the occasional blue colonies found on E.M.B. medium. It is suggested that these colonies form a relatively high pH and thus cause slight dissociation of the compound. This dissociation would allow independent staining of the colonies by methylene blue.

The use of eosin-methylene-blue (E.M.B.) agar as a differential medium in the colon-typhoid-dysentery group has become well established in bacteriological practice since its introduction by Holt-Harris and Teague (1916) and later modification by Levine (1918). The use of this medium, however, has been based on purely empirical considerations, and no explanation of the actual mechanism of differentiation has, to our knowledge, been offered.

Wilson (1907), in a review of differential staining by mixtures of eosin and methylene blue, has called attention to the formation of a compound by these two dyes. It was thought that such a compound might be, in part, responsible for the characteristic selective action of the medium. The work to be reported here was undertaken in an attempt to test this possibility and to determine any other contributing factors.

Materials

The following organisms[1] were chosen as being characteristic of the enteric group on eosin-methylene-blue agar:

1. *Escherichia coli communis*
2. *Aerobacter aerogenes*
3. *Aerobacter cloacae*
4. *Salmonella schotmülleri*
5. *Eberthella typhosa*
6. *Shigella paradysenteriae* Flexner

The basal medium for all cultural tests was prepared according to Levine's formula. This basal medium (minus lactose) was modified by omission or substitution of the various components so that the effect of the individual parts could be more readily studied. Both solid and liquid media were employed.

TABLE 1. ACID PRODUCTION ON AGAR AFTER 24 HOURS INCUBATION
Medium: E.M.B. basal formula* with dyes replaced by brom thymol blue

Organism	Medium 1 (glucose)	Medium 2 (lactose)	Medium 3 (no sugar)
Escherichia coli	acid	acid	alk.
Aerobacter aerogenes	acid	acid	alk.
Aerobacter cloacae	acid	acid	alk.
Salmonella schotmülleri	acid	alk.	alk.
Eberthella typhosa	acid	alk.	alk.
Shigella paradysenteriae Flexner	acid	alk.	alk.

*The E.M.B. basal formula as referred to in this and following tables is as follows:

Bacto-Peptone	1.0%
Dipotassium phosphate	0.2%
Eosin Y	0.04%
Methylene blue	0.0065%
Agar	2.0%

Sugars were used in 1.0% amounts thruout the work reported here and in subsequent tables. Brom thymol blue was employed in a concentration of 0.0016%.

[1]The organisms listed were secured from the stock culture collection of the Department of Bacteriology, University of Texas, and have been used for many years in laboratory work here. These cultures were chosen because their reactions have remained constant over a considerable period of time and the reactions are typical (according to Bergey's Manual) for the species or strain. It might be mentioned that the *Escherichia coli* used is negative to both sucrose and salicin.

Experimental

Due to the fact that organisms react specifically on eosin-methylene-blue medium according to their capacity to ferment lactose, we decided to investigate first the effect of final hydrogen-ion concentration on color deposition in the colonies.

To obtain some idea of the acid production by colonies of the test organisms under various conditions, media containing brom thymol blue were prepared, poured in Petri dishes, and the agar surface streaked from young broth cultures. The results are indicated in Table 1.

In general, Medium No. 1 served to show that in the presence of a fermentable carbohydrate (glucose) all the organisms produced an acid reaction. In the presence of lactose (Medium No. 2) the colon forms produced acid and the pathogens did not, while a uniform alkaline reaction resulted when all carbohydrate sources were removed (Medium No. 3).

Table 2. Acid Production in E.M.B. Broth

Organisms	pH
Escherichia coli	4.81
Aerobacter aerogenes	5.34
Aerobacter cloacae	5.60
Salmonella schotmülleri	8.24
Eberthella typhosa	7.68
Shigella paradysenteriae Flexner	7.24

To secure more quantitative results and gain some idea of the actual pH values involved, the eosin-methylene-blue medium was made up with 1.0% lactose, and with the agar omitted. The liquid medium was distributed in 10 cc. amounts in test tubes, inoculated with the test organisms and incubated at 37° C. for 24 hours. The pH of aliquot samples was then determined by means of a Beckman meter. The values secured are recorded in Table 2 and accord well with the results obtained from the brom-thymol-blue plates.

Knowing the relative degree of acidity under the various conditions of growth, it was next attempted to determine the effect of these various pH-values on the dyes when present independent of each other. For this determination six media were devised and streaked with the organisms. Results are recorded in Table 3.

These results indicate (Media No. 4 and No. 5) that methylene blue in the absence of eosin was not taken up by any of the organisms giving rise to an acid reaction, while Medium No. 6 showed that methylene blue colored all colonies not producing acid. Media

No. 7, No. 8, and No. 9 demonstrated the reverse activity in regard to eosin; for in the absence of methylene blue, eosin was taken up by all colonies fermenting the carbohydrates with resultant acid production. If no acid was elaborated, the eosin did not color the colony.

To obviate error due to color of the agar medium, colony color was determined by spreading a small amount of growth from each plate on white porcelain.

To determine the effect of the final hydrogen-ion concentration when the dyes are both present, media were prepared and inoculated by streaking the surface of the agar. The media and the results are presented in Table 4.

TABLE 3. COLONY COLOR ON VARIOUS DYE AND CARBOHYDRATE COMBINATIONS AFTER 24 HOURS INCUBATION

Organism	Basal formula without eosin			Basal formula without methylene blue		
	Medium 4 (glucose)	Medium 5 (lactose)	Medium 6 (no sugar)	Medium 7 (glucose)	Medium 8 (lactose)	Medium 9 (no sugar)
Escherichia coli	no color	no color	blue	red	red	no color
Aerobacter aerogenes	no color	no color	blue	red	red	no color
Aerobacter cloacae	no color	no color	blue	red	red	no color
Salmonella schotmülleri	no color	blue	blue	red	no color	no color
Eberthella typhosa	no color	blue	blue	red	no color	no color
Shigella paradysenteriae Flexner	no color	blue	blue	red	no color	no color

Reference to Table 4 (Medium No. 10) will show that in the presence of a fermentable carbohydrate all of the organisms were colored by the eosin-methylene-blue mixture, and this medium did not serve to differentiate pathogens from *Escherichia coli*. The characteristic metallic sheen was present on all except the *Aerobacter spp.* Medium No. 11 gave the familiar distinction between colon and pathogenic forms. Medium No. 12, like Medium No. 10, did not serve to differentiate between colon forms and pathogens, but for a different reason. Whereas in Medium No. 10 all of the organisms produced acid and all colonies were colored, in Medium No. 12 none of the organisms produced acid and none was colored.

Furthermore, Media No. 13 and No. 14 were examined just at

the time when normal eosin-methylene-blue plates first developed typical *E. coli* colonies. At this time both the brom-cresol-purple plates and the methyl red test indicated an acid reaction. In the case of *Aerobacter* forms, a similar correlation between acid reaction and initiation of color absorption was found, altho the degree of acidity was not as high. It will be seen that these tests indicate a pH-range on solid media quite similar to that shown in Table 2 for liquid medium.

The results of this experiment indicate that the production of a colored colony on eosin-methylene-blue medium is a function of acid production, and the color of the colon type colonies is not shown

TABLE 4. COLONY COLOR ON E.M.B. MEDIUM AT DIFFERENT pH-VALUES AFTER 24 HOURS INCUBATION

Medium: E.M.B. basal formula. Dyes omitted in Media 13 and 14.

Organism	Medium 10 (glucose)	Medium 11 (lactose)	Medium 12 (no sugar)	Medium 13 (lactose) Brom cresol purple	Medium 14 (lactose) Methyl red
Escherichia coli	typical*	typical	no color	acid	acid
Aerobacter aerogenes	typical	typical	no color	acid	acid
Aerobacter cloacae	typical	typical	no color	not run	not run
Salmonella schotmülleri	like *E. coli*†	typical	typical	alk.	alk.
Eberthella typhosa	like *E. coli*	typical	typical	alk.	alk.
Shig. paradysenteriae Flexner	like *E. coli*	typical	typical	alk.	alk.

*"Typical" refers to characteristic appearance on Levine's E.M.B. agar.
†"Like *E. coli*" refers to characteristic appearance of *E. coli* on Levine's E.M.B. agar.

until an acid reaction has been produced by action of the organisms on the substrate.

This coloration of the acid-producing colonies is, of course, the basis for the differential value of eosin-methylene-blue agar. An extremely important point in explaining this differential action of eosin-methylene-blue medium, and one which has received insufficient attention in the literature, is the fact that non-lactose-fermenting organisms produce *colorless* colonies. This is entirely at variance with what might be expected, for the pH of the substrate is in the alkaline range and a basic dye (methylene blue) is present which, according to the well-known theory of Stearn and Stearn (1924),

should be effective under these conditions. The results recorded in Table 3 (Medium No. 5) indicate that this contention is valid, for when eosin is omitted from the eosin-methylene-blue medium, the methylene blue is taken up by non-fermenting organisms.

Furthermore, if the dyes act independently in the eosin-methylene-blue medium we should expect the acid dye, eosin, to be taken up by colonies of fermenting organisms because of the resultant acid reaction of the substrate. When eosin alone is present such is the case; but even a cursory examination of colon type colonies on eosin-methylene-blue agar indicates that something more than eosin is responsible for the colony coloration. This extra color cannot come from independent action of methylene blue, for it has been shown in Table 3 (Medium No. 5) that methylene blue is not taken up by the lactose fermenters.

Since methylene blue should result in blue colored colonies of lactose non-fermenters but does not, and since lactose fermenters are colored by something more than eosin and which apparently is not referable to free methylene blue, it is immediately suggested that we are dealing with something other than a simple mixture of the two dyes. The results detailed above would become explicable if eosin and methylene blue formed a compound; for, in eosin-methylene-blue medium, eosin is present in considerable molecular excess. Thus, the methylene blue would be completely prevented from independent staining activity as long as it remains in the combined state.

If this is true, eosin-methylene-blue medium modified so as to contain a molecular excess of methylene blue should result in blue colored lactose non-fermenters, and in the case of lactose fermenting organisms the colonial color should be essentially normal. To test this possibility such a medium was made and streaked with the test organisms. The details are presented in Table 5.

It must be noted that this eosin-methylene-blue compound acts as a weakly acid or neutral dye, for if this were not true it could not color the acid-producing organisms on Media No. 10, No. 11, and No. 15, but would color the colonies of organisms which do not produce acid. Adoption of the technic of Clifton and Madison (1931), for determining the nature of the charge on particles, showed the eosin-methylene-blue compound to spread as far on filter paper as the solvent. The compound was thus shown to be composed of negatively charged particles, as are all acid dyes. Further evidence that the compound is not basic in nature was obtained by filtering separately thru Berkfeld candles: eosin-methylene-blue liquid

medium; broth containing only methylene blue dye; and broth containing only eosin. In all cases the dyes were in the proportion of Levine's formula. The compound was passed thru the candle at a rate comparable to that of eosin alone, while the basic methylene blue was withheld for an appreciably longer time. This would indicate either an acid or neutral dye. It might be noted here that either an acid or a neutral dye would give rise to the phenomena recorded.

The action of this compound in staining bacterial cells is of interest. A concentrated mixture of the two dyes was prepared maintaining Levine's ratio, but securing a concentration 25 times stronger. Above pH 6.5 (approx.) staining was very faint, and at pH values above 7.3 this solution entirely failed to stain films of *E. coli*. A control stain, however, made with methylene blue in equivalent

TABLE 5. COLONY COLOR AFTER 24 HOURS ON E.M.B. AGAR CONTAINING A MOLECULAR EXCESS OF METHYLENE BLUE*

Medium: E.M.B. basal formula with methylene blue concentration increased to 0.022%

Organism	Medium 15 (lactose)
Escherichia coli	typical
Aerobacter aerogenes	typical
Aerobacter cloacae	typical
Salmonella schotmülleri	blue
Eberthella typhosa	blue
Shigella paradysenteriae Flexner	blue

*Assuming one molecule of methylene blue to one of eosin.

concentration, resulted in a definite blue stain. All films were stained for 5 minutes at room temperature.

For the purposes of the eosin-methylene-blue reaction, the compound may be considered an acid or neutral dye, for, as shown in Tables 2 and 4, all lactose non-fermenters gave a final pH above the range 6.5–7.3 and all lactose fermenters gave a lower pH.

In an effort to determine whether colony color is due to actual staining of the cells or merely to deposition of color in the interstices between the cells in the colony, growth of *E. coli* from eosin-methylene-blue agar was examined microscopically and the cells found to be stained. Cells suspended in water of approximately the same pH as that attained on plates and then centrifuged gave no evidence of release of the stain. Identical results were obtained

when this procedure was applied to *E. coli* from a medium containing only the acid dye, eosin. The color of the eosin-stained cells was, of course, somewhat different from those stained with the eosin-methylene-blue compound.

Additional evidence of a more chemical nature for the actual formation of a methylene blue eosinate was obtained by precipitation of the compound with *N*/1 HCl. A solution of methylene blue and eosin in the concentrations employed in Levine's formula and buffered with 0.2% K_2HPO_4 yielded a copious precipitate at pH 4.7, at which hydrogen-ion concentration either dye alone is perfectly soluble. Eosin precipitated in mildly acid solutions, but the precipitate was entirely dissolved on back-titration with NaOH to pH 4.1. These pH-values were carefully determined on a Beckman pH-meter and the results repeatedly confirmed.

The actual nature of the compound precipitated at pH 4.7, which is the approximate hydrogen-ion concentration reached by *E. coli* on eosin-methylene-blue medium, was next investigated. Since

TABLE 6. TITRATION OF SAMPLES OF THE EOSIN-METHYLENE-BLUE COMPOUND

Weight of sample	N/100 $TiCl_3$ for M.B. reduction	Amount of M.B. present	Eosin (by difference)	Ratio MB/E (found)	Ratio MB/E (theoretical)
19.3 mg.	4.0 cc.	5.7 mg.	13.6 mg.	0.42	0.41
30.2 mg.	6.5 cc.	9.2 mg.	21.0 mg.	0.43	

direct precipitation does not yield a sufficiently coarse precipitate, the following procedure was followed: 10.0 cc. of *N*/1 HCl was added to 500 cc. of the buffered solution of the dyes described above and this was then back titrated to pH 4.7 with NaOH. The precipitate was collected by filtration thru a Buchner funnel, washed thoroly with *N*/500 HCl, and dried to constant weight at 110°–120° C. Weighed samples, dissolved in acetic acid and alcohol, were then titrated for methylene blue content by reduction with *N*/100 $TiCl_3$ in an atmosphere of CO_2 according to the method of Holmes (1929).

Uncombined methylene blue was found to require two molecules of $TiCl_3$ for the reduction of one molecule of dye, and from this the actual amount of methylene blue in the compound was readily calculated. The molecular weight of methylene blue was taken to be 284, since it was postulated that methylene blue lost one atom of chlorine in forming the eosinate. The amount of eosin in the compound was found by difference.

The results tabulated in Table 6 show that the experimentally

determined ratio of methylene blue to eosin in the compound agrees closely with the theoretical for a compound containing one molecule of each dye.

It is possible, from the above considerations, that the compound may be formed by splitting out NaCl between the carboxyl group of a molecule of the sodium salt of eosin and a molecule of methylene blue chloride. Employing the formulas for these two dyes as given by Conn (1940), such a compound would have an -ONa group attached to a benzene ring. In acid solution this would give a phenolic group, and would account for the precipitation of the compound.

Occasionally, blue colonies are encountered on eosin-methylene-blue plates in water analysis. In an effort to explain this, eosin-methylene-blue agar was prepared and adjusted to a relatively high pH, the organisms streaked on the agar surface and incubated at 37° C. Results are given in Table 7.

TABLE 7. COLONY COLOR ON E.M.B. AGAR AT pH 8.0–8.4

Organism	Incubation period (hours)	Medium No. 16
Escherichia coli	24	typical
Aerobacter aerogenes	24	typical
Aerobacter cloacae	24	typical
Salmonella schotmülleri	24	blue
Eberthella typhosa	24	blue
Shigella paradysenteriae Flexner	24	blue

Methylene blue could be taken up, as was shown, if the compound dissociated to some extent at the alkaline pH. This would produce a small amount of free methylene blue, which would be taken up by the non-lactose-fermenting organisms, thus displacing the equilibrium in the direction of further dissociation.

CONCLUSIONS

1. The production of a colored colony on eosin-methylene-blue agar is a direct function of acid production.

2. Evidence is presented to show that eosin and methylene blue do not exist as a simple mixture in eosin-methylene-blue medium, but react to form a compound which is in the nature of an acid or neutral dye.

3. The compound formed at an acid reaction is composed of one molecule of methylene blue and one of eosin.

4. The color of acid-producing colonies on eosin-methylene-blue

medium is referable to actual staining of individual cells with this compound.

5. The formation of the compound provides a basis for the explanation of the differential action of the medium. A possible explanation is offered for the production of occasional blue type colonies encountered on the medium.

The authors wish to express their gratitude for the help and advice offered by Dr. I. M. Lewis during the course of this work.

REFERENCES

CLIFTON, C. E., and MADISON, R. R. 1931. Studies on the electrical charge of bacteriophage. J. Bact., **22**, 255–60.

CONN, H. J. 1940. Biological Stains, *4th Ed.* Biotech Publications, Geneva, N. Y.

HOLMES, W. C. 1929. The chemical analysis of thiazine eosinates. Stain Techn., **4**, 49–52.

HOLT-HARRIS, J. E., and TEAGUE, O. 1916. A new culture medium for the isolation of *Bacillus typhosus* from stools. J. Inf. Dis., **18**, 596–600.

LEVINE, MAX. 1918. Differentiation of *B. coli* and *B. aerogenes* on a simplified eosin-methylene blue agar. J. Inf. Dis., **23**, 43–7.

STEARN, E. W., and STEARN, A. E. 1924. The chemical mechanism of bacterial behavior. J. Bact., **9**, 463–77.

WILSON, THOMAS M. 1907. On the chemistry and staining properties of certain derivatives of the methylene blue group when combined with eosin. J. Exp. Med., **9**, 645–70.

A NEW MICROCHEMICAL REACTION FOR CELLULOSE

E. E. Post and J. D. Laudermilk, *Pomona College, Claremont, Cal.*

Abstract.—A microchemical test for cellulose applicable to fresh sections and commercial products is described. The test differs from the older technics in that materials tested are not permanently altered.

Two solutions are required: (1) 2% solution of iodine in 5% KI, diluted with 9 parts by volume of water containing 0.28% glycerin; (2) saturated aqueous LiCl.

Procedure: Apply 2 or 3 drops of solution 1 with a glass rod; allow the preparation to stand for 30 sec.; blot with filter paper, drying as completely as possible. Apply one drop of solution 2, cover and examine. The color reaction will be obtained within 5 min. The reaction for pure cellulose is light blue. Reactions for 16 fibers are given in the table.

As a stain for demonstrating plant tissues the technic has been used in the Botany Department of Pomona College with much success; but this phase of the subject has not been extensively investigated.

The microchemical identification of cellulose is sometimes required in police work and not infrequently in the testimony of the expert witness and technical referee in civil cases. The importance of this identification to the student of vegetable histology is self evident. The determination, as generally carried out, depends on the color produced after the material under examination has been treated with iodine in conjunction with a second reagent. Perhaps zinc chloroiodide applied as a single solution (Herzberg's reagent) is the most generally accepted procedure. Cross and Bevan (1900, pp. 95–110) advocate the use of glycerin-sulfuric-acid and iodine as two solutions. Mangin (1901, p. 141) recommends a solution of iodine in $CaCl_2$. Sutermeister (1929, pp. 467–8) indorses the use of $CaCl_2$ and iodine as two solutions.

In any one of these cases, the specific iodine reaction is only obtained after the cellulose has been altered by the action of the second reagent.

Authorities are not altogether in agreement regarding the color reactions for commercial fibers when treated with the same reagent.

The following table, compiled from several sources brings this point forward:

TABLE 1. IODINE REACTIONS OF CELLULOSE AND COMMERCIAL FIBERS

Fiber	Color reactions with reagents indicated					
	I, H_2SO_4[1]	I, $ZnCl_2$[2]	I, $ZnCl_2$[3]	I, $ZnCl_2$[4]	I, $ZnCl_2$[5]	I, $CaCl_2$[6]
Cotton	blue	wine red	wine red	violet	wine red	red to brownish red
Flax	blue	purple to yellow	wine red, yellow		wine red	red to brownish red
Hemp	green	purple to yellow			yellow, yellow-green	red or brownish
Jute	yellow-brown	yellow	yellow to lemon		yellow, yellow-green	greenish
Manila	yellow	yellow	yellow to lemon		yellow	greenish
Manila, cooked		red	wine red		blue	
Straw		blue, greenish			yellow	
Esparto	blue and yellow	blue, green	blue, navy		blue	
Ramie	blue, violet	dark red, green, yellow	blue			red or brownish red
N. Z. flax	deep yellow-brown	deep yellow, brownish				

It is conceivable that under certain conditions, advantage might be taken of this disagreement of the authorities. In view of this apparent deficiency, a procedure which is simple, precise, absolutely dependable, and one whereby the material under examination would not be permanently altered seemed desirable.

After extended investigations of the material present in fossil vegetable remains[7] and other researches where these identifications

[1]Cross and Bevan (1900), pp. 95–110
[2]Mathews (1936), pp. 339–42
[3]Sutermeister (1929), pp. 466–67
[4]Zimmermann (1901), pp. 140–41
[5]Chamot and Mason (1930), pp. 457–58, Vol. I.
[6]Sutermeister, loc. cit. pp. 467–68
[7]Laudermilk and Munz (1938) pp. 271–81.

are of importance from a criminological standpoint[8] we have developed the following technic which has all the advantages and none of the defects sometimes experienced with older methods. The procedure requires the application of two solutions:

Solution A. Dilute iodine. Prepare a 2% solution of iodine in 5% KI. Dilute 20 cc. of this solution with 180 cc. distilled water; add 0.5 cc. glycerin and mix by shaking.

Solution B. Saturated solution of LiCl. Saturate 15 cc. distilled water with LiCl at 80° C.; cool to room temperature, and use the supernatant solution.

Application. To the teased out fibers or section, apply 2 or 3 drops of the iodine solution by means of a glass rod. Allow to stand for 30 seconds; longer standing does not effect the reaction. Blot with a piece of filter paper; the sample should be as dry as possible; the presence of the trace of glycerin insures that the fibers or section will remain active toward the second reagent. Add a drop of the LiCl solution, cover and examine. The color reaction will be obtained within 5 minutes. Color reactions with these reagents are shown in Table 2:

TABLE 2. REACTIONS OF CELLULOSE AND COMMERCIAL FIBERS WITH IODINE AND LITHIUM CHLORIDE

Typical color	Fiber
Light blue	cotton, soda pulp, bleached sulfite, straw, esparto
Dark blue	pineapple fiber
Greenish blue	linen
Green to yellowish green	sisal, manila hemp, yucca
Yellow	yucca, ground wood, manila hemp, hemp
Lemon yellow	kapok
Brownish yellow	jute

Applied as a differentiating stain to plant sections in the study of vegetable histology, the LiCl and iodine method has been used with much success in the Pomona College Botany Department. This phase of the subject is one which might well be investigated further by students whose interests lie more particularly in this field.

Altho transverse sections of cotton and other fibers treated with these reagents show that the fiber is stained equally thruout, irrigation of the mount with water immediately discharges the stain. The reagents may again be applied with identically reproducible results.

[8]Laudermilk (1933) pp. 503–16.

REFERENCES

CHAMOT, E. M., and MASON, C. W. 1930. Handbook of Chemical Microscopy, Vol. 1. John Wiley and Sons, New York.

CROSS, C. F., and BEVAN, E. J. 1900. A Text-Book of Paper Making. Spon and Chamberlain, New York.

LAUDERMILK, J. D., and MUNZ, P. A. 1938. Plants in the Dung of Nothrotherium from Rampart and Muav Caves, Arizona. Carnegie Institution of Washington Publication No. 487, pp. 271–81.

LAUDERMILK, J. D. 1933. The identification of cloth ash. J. Criminal Law and Criminology, 24, 503–16.

MANGIN, LOUIS. 1888. Sur les réactifs jodes de la cellulose. *Cited from* Zimmermann (1901).

MATHEWS, J. MERRITT. 1936. The Textile Fibers, *4th Ed.* John Wiley and Sons, New York.

SUTERMEISTER, EDWIN. 1929. The Chemistry of Pulp and Paper Making, *2nd Ed.* John Wiley and Sons, New York.

ZIMMERMANN, DR. A. 1901. Botanical Microtechnique. Henry Holt and Co., New York.

A RAPID DETERMINATION OF CONCENTRATION IN LIQUID-IN-LIQUID SOLUTIONS

LOUIS K. MANN AND HAROLD L. JETER, *Ohio University, Athens, Ohio*

In the microtechnical laboratory it is frequently convenient to have available a rapid method for determining the approximate concentration of aqueous alcoholic or other solutions. For example, purchased dye solutions occasionally are not labeled as to alcoholic content, or one may desire to check quickly the strength of alcohols which have stood in bottles or stenders for some time.

The rapid agitation resulting from surface tension differences when alcoholic solutions of different concentrations are mixed on a microslide has given rise to the following method. This procedure may be applied to a number of liquid-in-liquid solutions, and has simplicity and rapidity to recommend it. It is commonly known that when a drop of a liquid is placed on the surface of a thin layer of a second liquid with which it is miscible, the behavior of the drop depends upon the relative surface tensions of the two liquids. The drop of liquid may: 1) spread rapidly, thinning the layer at the point of application until a noticeable depression results; 2) remain as a convex drop for some time before spreading; or 3) mix uniformly with the second liquid.[1]

In the case of miscible liquids, the interfacial tension of the drop and the layer of liquid may be neglected so that the relative air-liquid surface tension of the two liquids determines the reaction of the drop. If the drop has a lower surface tension than the layer on which it is placed, the immediate spreading of the drop occurs. If the drop has a higher surface tension than the liquid layer, it maintains a distinct convex upper surface for several seconds before spreading. If the two liquids have equal surface tensions, they mix without marked surface disturbances.

Suppose one has a solution (call it "solution A") made up of two liquids which differ widely in surface tension—such as water and ethyl alcohol. Pure ethyl alcohol has a low surface tension (approximately 22 c. g. s. units), while the surface tension of water is high (approximately 73 c. g. s. units). The surface tension of solution A lies somewhere between these two values, and varies with the percentage of alcohol present. Now by comparing the surface

[1]Willows, R. S., and Hatschek. 1923. *Surface Tension and Surface Energy.* P. Blakiston's Son & Co., Philadelphia.

tension of solution A with that of solutions of known alcoholic content, the concentration (alcoholic percentage) of solution A may be approximately determined by the following method:

Spread a few drops of 50% alcohol on a slide and add a drop of solution A. If the drop remains convex for several seconds before spreading, the surface tension of solution A is higher than that of 50% alcohol, i. e., alcohol in solution A is less than 50%. Repeat with a solution of perhaps 30% alcohol. If rapid spreading of the drop occurs, a lower surface tension of the drop is indicated, i. e., solution A has more than 30% alcohol. Continue with intermediate concentrations until no marked surface activity is seen. The solution giving this is approximately of the same alcoholic concentration as solution A. This process is not as dependent on trial and error as seems indicated, since the intensity of the reaction is a good indication of the relative difference in alcoholic concentration. This has been checked with solutions of known concentration varying from pure water to absolute alcohol. Thruout this range in concentrations, a 2% difference gives a definite reaction.

To observe the surface layer, the slide can be held about a foot below and to the side of a 60 watt Mazda frosted bulb so that the reflection of the bulb is seen on the surface of the liquid. Unequal heating of the solutions must be avoided since the surface tension changes with a change in temperature. It is essential to have only a thin film of liquid on the slide. For this reason, especially with highly aqueous solutions, slides must be free from grease. Heating in chromic acid cleaning solution is recommended, after which slides may be rinsed and placed in 70% acid alcohol until used. Rubber bulb pipettes used should also be carefully cleaned. As an additional precaution, the reaction must be completed rapidly because of changes in surface tension on evaporation or uptake of atmospheric moisture. With a little practice the test may be completed in less than 15 seconds.

The advantages of this method are: 1) it is very rapid; 2) it requires little material (often only a few drops); 3) it requires apparatus (clean slides and dropping pipettes) and standard solutions (known water-alcohol series) which are frequently on hand in the microtechnical laboratory; 4) it is sufficiently accurate for most microtechnical work (e. g., dye solutions and concentration series).

This method may be extended to various mixtures of liquids which differ widely in surface tension, such as aqueous solutions of acetic acid or acetone. Alcoholic solutions of various solids, such as dyes, may be checked for percentage of alcohol if care is used to avoid soaps or other substances which in small quantities have a marked effect on surface tension.

THE STAINING OF THE METACHROMATIC GRANULES IN CORYNEBACTERIUM DIPHTHERIAE

HARRY E. MORTON AND ALICE FRANCISCO, *Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*

ABSTRACT.—**The metachromatic granules, which sometimes are regarded as characteristic of the diphtherial species, are more easily differentiated when the basic dye is made up in a solution of which the reaction is quite acid. Of the usual stains employed for demonstrating the granules, the Neisser stain proves to give the best results.**

When the reduction of potassium tellurite by *Corynebacterium diphtheriae* was studied with the electron microscope,[1] it was observed that these organisms, growing on ordinary blood agar, showed in electron micrographs opaque spheres or plates which might correspond to the so-called polar granules. Smears, prepared from the same cultures and stained with Loeffler's methylene blue and examined by the oil immersion lens and ordinary light illumination, showed typical metachromatic granules within the diphtherial cells. During the investigation into the nature of these opaque spheres or plates, which appear in the electron micrographs, there was occasion to try the various stains recommended for demonstrating the granules within diphtherial cells. Such great differences in the results were obtained with the various stains on the same culture that we looked for a possible explanation; and certain conclusions were drawn which can now be reported. No attempt will be made to give a full explanation of the staining reactions of the so-called polar granules, but a note on an apparently significant phase in the technic for their staining is offered.

It is known that a medium containing blood or blood serum favors the production of granules within diphtherial cells; also that granules do not appear within the cells until the culture is several hours old. Consequently, cultures were grown on blood infusion agar (5% horse blood, 1% peptone, from Parke, Davis & Co.) at 37° C. for about 48 hours. Numerous strains of *C. diphtheriae*, *C. pseudodiphthericum*, and *C. xerosis* were studied. The stains, with the exception of

[1]Morton, H. E., and Anderson, T. F. 1941. Electron microscopic studies of biological reactions. I. Reduction of potassium tellurite by *Corynebacterium diphtheriae*. Proc. Soc. Exp. Biol. and Med., 46, 272–6.

Neisser's, were prepared according to the formulae given in The Manual of Methods for Pure Culture Study of Bacteria, Leaflet IV, 7th edition.[2] Neisser's stain was prepared according to the formula of Kolmer and Boerner.[3]

The following stains were used in the examination of the cultures for granules: Loeffler's methylene blue, prepared with 0.01% KOH; Loeffler's methylene blue, prepared with distilled water; Ponder's; Neisser's; Beck's; Albert's; and Mallory's. The results obtained with a given culture varied from cases where there were no visible granules to those with a high percentage of the cells showing granules. Such discrepancies warranted investigation, and observations made while applying the differential staining technic (0.01% toluidine blue in a buffered solution, followed by 0.01% fast green FCF in a similar solution) described by Kelley[4] offered a possible explanation. With this latter technic it was observed that in the very acid range (pH 1.9) some of the granules retained the toluidine blue whereas the remainder of the cell stained with the fast green. As the reaction of the staining solutions became more alkaline than about pH 3.4, the cells retained more of the toluidine blue and less of the fast green, and the granules were not as easily discernible. This suggested that a good staining solution for the granules would be one in which the basic dye such as toluidine blue or methylene blue was in a solution having a pH below 3.5. The reaction of the various staining solutions used for demonstrating the granules was determined with a Leeds and Northrup Glass Electrode. The results are shown in Table 1.

TABLE 1. THE pH OF THE SOLUTIONS OF THE STAINS COMMONLY EMPLOYED FOR DEMONSTRATING GRANULES WITHIN DIPHTHERIAL CELLS

Stain	pH
Albert's	2.8+
Mallory's	2.82
Neisser's, solution I	2.82
Neisser's, solution II	3.2
Ponder's	3.22
Beck's, solution I	3.65
Beck's, solution II	3.55
Loeffler's methylene blue (aqueous)	5.5
Loeffler's methylene blue (alkaline)	8.3

[2]Biotech Publications, Geneva, N. Y. 1939.

[3]Kolmer and Boerner. 1941. Approved Laboratory Technic, *3rd Ed.* D. Appleton-Century Co., New York.

[4]Kelley, E. G. 1939. Reactions of dyes with cell substances. V. Differential basic dye combination of tissue nuclei with special reference to resting and mitotic cells of tumor tissue. J. Biol. Chem., **127**, 73–86.

Neisser's stain was found to be the best for demonstrating the granules. The dark blue granules were very easily discernible, in contrast to the light brown cells. Albert's and Mallory's stains were very good; Ponder's stain also gave good results but has the drawback that it is a wet mount and the preparations cannot be preserved. Beck's stain was not quite as good as Neisser's. Least effective of those tried was Loeffler's alkaline methylene blue.

Since staining of the granules within the diphtherial cells involves chemical reactions between dyes and bacterial cell substances, one would expect that the results would depend not only on the affinity of dye for cell substance but also on the pH at which this reaction is allowed to take place. This latter assumption has been borne out by the observations cited above.

STAINING INVERTEBRATE BLOOD FOLLOWING MAXIMOW'S OSMIC ACID FIXATION

EMIL LIEBMANN, *Division of Microscopic Anatomy, Tulane University, New Orleans, La.*

Aided by a grant from the Dazian Foundation for Medical Research.

Maximow's[1] fixative is doubtless one of the best of those containing osmic acid, as it does not reduce the staining properties and enables the application of a variety of stains. It gives very good results with blood stains, and this is of considerable importance for invertebrate work, where fat-carrying lymphocytes are very common.

Unfortunately, invertebrate material—or at least the groups studied, annelides and insects—give relatively poor results even with Maximow's azure-eosin modification for cold blooded vertebrates. The sections appear overstained with azure and consequently too basophilic.

Extended differentiation, as recommended by Romeis[2] for vertebrates, produces better results but renders the sections so pale as to make them useless. This invariably applies if neutralized water is used for diluting the stain. Ordinary distilled water gives good color differentiation of the tissues; but the nuclei are usually poorly outlined as they differ little in color from the cytoplasm and their elements (chromatin, nucleolus) show no color differences.

Romanovsky stains (Wright's and Leishmann's were used) appear unsuitable for invertebrate material fixed with Maximow's OsO_4 when applied in the manner practised in vertebrate work. They produce a uniform blue coloration without any indication of eosinophils.

The application of a slightly acidulated liquid for differentiation has been found to surmount these difficulties and enables the use of Wright's or Leishmann's stain.

Staining with azure-eosin is accomplished as follows: add one drop of a 5% aqueous solution of acetic acid to each 25 cc. of 95% alcohol used for differentiation (sections should be well overstained and neutral water used for diluting the stock solutions); differentiate

[1]Maximow, A. 1909. Über zweckmässige Methoden für cytologische und histogenetische Untersuchungen am Wirbeltierembryo. Zts. wiss. Mikr., 26, 177–90.

[2]Romeis, B. 1928. Taschenbuch der mikroskopischen Technik, *12th Ed.* Oldenburg, Munich & Berlin.

0.5–1 minute; absolute alcohol, ½–1 minute; xylene, 3 changes; mount.

Romanovsky stains are in many instances superior to Maximow's azure-eosin. This is particularly the case in the study of lymphocytes.

The procedure developed is somewhat different from that used in vertebrate work, being as follows: (1) Stain 24 hr. in a strong solution of Wright's or Leishmann's stain (1 cc. of the stock solution for each 50 cc. of H_2O). (2) Differentiate 1–1½ min. in acidulated H_2O, (1 drop of 5% acetic acid: 25 cc. of water). (3) Dehydrate in acetone, 30 sec. (4) Xylene, (3 changes). (5) Mount in liquid paraffin or Clarite.

A pale appearance of the sections is usually an indication of insufficient overstaining. In such cases the solution should be made stronger, up to 2 cc. of the stock for each 50 cc. of water. The differentiation is then somewhat prolonged.

The method of applying a slightly acidulated medium for differentiation was mostly applied to sections; but wet smears fixed in Maximow's OsO_4 fluid produce similar results.

In conclusion it should be stressed that differentiation in the slightly acidulated liquids does not affect the various types of granulations found in invertebrates.[3]

[3]Dr. William Bloom, University of Chicago, is to be thanked for furnishing some of Maximow's original vertebrate slides, for comparison.

FLAGELLA STAINING OF ANAEROBIC BACILLI

ELIZABETH O'TOOLE, *Department of Bacteriology, University of Colorado School of Medicine and Hospitals, Denver, Colo.*

ABSTRACT.—An improved technic for staining flagella of anaerobic bacilli is described. This involves the use of the flagella stain described by Bailey, and careful attention to several details. Those considered of most importance are listed, as follows:

1. At least two rapid subcultures were made in broth containing peptone, meat infusion and glucose; the broth was centrifugalized and the cells washed in sterile distilled water before inoculation upon anaerobic agar slants.

2. All distilled water used was sterilized by passing thru a sterile Mandler filter to remove precipitate; the temperature of the water ranged from 30° to 37° C., and the pH was approximately 6.8.

3. The harvested cells were incubated at 37° C. one-half hour before staining to obtain better distribution.

4. A small drop of suspension was placed near one end of specially prepared slides, allowed to flow slowly over the surface, and placed in the incubator, without heating, in a slanted position to dry.

5. Freshly made and freshly filtered solutions were always used, and the stains washed off with distilled water blown gently thru a wash bottle.

6. With the use of carbol fuchsin, careful experimentation was required as to the amount of heat necessary to stain the flagella. The limits of time appeared to be between one-half and one minute. The slides were air-dried, and not blotted.

7. In the writer's hands, the following experiment has proved of value for the staining of flagella of pathogenic bacteria where ordinary methods have failed. A three year old stock culture of a pathogenic strain of *Bacillus difficilis* was used. A guinea pig was injected subcutaneously with 2 cc. of a 48-hour brain-broth culture, and the bacillus recovered at autopsy. With careful and appropriate microscopical and cultural methods the bacillus was found to correspond to the original description, and pathogenicity for another guinea pig proven. These cells produced satisfactorily stained flagella, whereas the control slides, using the stock culture, showed rods with few and faintly stained flagella.

The literature on flagella staining contains many references to the staining of flagella of bacterial species in general, but few describe

the cultural and technical difficulties encountered in staining flagella of the anaerobic species in particular. An interesting and valuable description of a comprehensive investigation of this problem is by Levenson (1936) who modified the flagella stain of Casares-Gil. This consisted mainly in substituting basic dyes for the impregnation of silver salts, and a cultural technic involving the growth of the anaerobes in broth in sealed capillary tubes.

The encouraging results obtained by the writer with the flagella staining of aerobic bacteria as described by Bailey (1929) furnished the impetus for more extensive investigation with anaerobic species in spite of discouraging and indifferent results with other stains, particularly the silver impregnation methods.

Many modifications and variations were tried to obtain the most satisfactory preparations. The writer does not feel, however, that these results were outstanding enough for extensive discussion here, but in the course of the investigation much information was obtained as to cultural and staining methods, and technics, which vary in some particulars from the usual published methods. Out of this work was evolved the following schedule for the staining of anaerobic bacilli.

Cleaning Slides

The following technic was found to be very satisfactory for obtaining slides that were absolutely clean and free from grease:

1. Immerse preferably new slides or slides without scratches for a few hours in a cleaning solution consisting of 10% $K_2Cr_2O_7$ (commercial), dissolved in concentrated H_2SO_4 (commercial).
2. Rinse in running tap water.
3. Wash in hot, soapy water, rinse thoroly in running tap water, and finally in distilled water.
4. Put immediately into a solution of equal parts distilled water and 95% alcohol acidified by adding 1 cc. HCl to 100 cc. of solution.
5. Just before using remove the slides individually with forceps, immerse in 95% alcohol and touch to a flame; then place upright on a clean surface. This avoids the necessity of drying with a towel and handling with the fingers.

No glass or lead pencil marks should be put on the slides before staining, as these may come off and spoil the stain. If desired, marking may be done with a diamond point marking pencil.

Cultures

The writer agrees with other workers in this field that the age of the culture is very important, and that young cultures contain a higher proportion of actively motile cells than older ones.

The strains of anaerobic bacilli used came from stock cultures.[1] Some of these had been sealed off for 2 or 3 years, and one or two as long as 6 years. Experience showed that better results were obtained if rapid transplants of the bacilli were made before making the flagella stains. This was carried out in the following manner: The cultures were inoculated into boiled and cooled glucose (1%) beef heart infusion broth (pH 6.8) in constricted tubes with marble seal (Hall, 1921). Every 6 hours, or as soon as growth was visible, transplants were repeated as described above. Two, sometimes three, successive transplants were required. This was determined by microscopic examination under coverslip preparation for motility. At this time the cultures were examined for the presence or absence of aerobic contaminants by subculture on agar slants.

Liquid media were tried for flagella staining with indifferent success; best results were secured from growth on solid media. Blood agar slants were inoculated anaerobically by the following method. Actively growing cultures in constricted tubes of glucose broth with marble seal, as described above, were centrifugalized for 10 minutes. The broth was drawn off with a pipette and the sediment resuspended in a few cubic centimeters of sterile distilled water. About 0.1 cc. of this was dropped onto the blood agar slants in glass test tubes size 15 × 150 mm., and allowed to cover the surface. The cotton plug was cut off level with the mouth of the tube, and was then inverted in a sterile larger tube, 23 × 200 mm. The cotton plug of this larger tube was cut off, leaving a plug of about one inch thickness, which was pushed down as far as possible, and pyrogallic acid (U.S.P., J. T. Baker Co.) in crystal form packed into the top with enough 10% NaOH solution (commercial) to liquefy the pyrogallic acid leaving a few cubic centimeters excess moisture. A No. 4 rubber stopper was inserted, the tube inverted immediately to prevent pyrogallic acid and NaOH from running down the inner tube, and incubated at 37° C.

When visible signs of growth appeared (from 11 to 36 hours, as shown in Table 1) the culture was opened and distilled water (approximately pH 6.8 and temperature 30° to 37° C.), sterilized by passing thru a sterile Mandler filter to remove most of the precipitate, was carefully pipetted over the surface. The tube was gently rotated to wash off the growth and when the liquid was fairly turbid it was carefully drawn off with a pipette into a small sterile tube. At this time the suspension was checked for pH, examined for motility by coverslip preparation, and the results recorded. The

[1]Stock culture collection of I. C. Hall.

suspension was then incubated at 37° C. for half an hour to obtain a more even distribution of the organisms. A drop of this was placed near one end of the slide and allowed to flow very slowly over the surface. A fine Pasteur pipette was used for this purpose. The slide was then placed in the incubator, without heating, in a tilted position to dry, because it is better if this is done as quickly as possible.

Staining

The stain described by Bailey (1929) was used without modification, and was prepared as follows:

Mordant:

5% aqueous tannic acid[2] ..... 75 cc.
10% aqueous $FeCl_2 \cdot 6H_2O$[2] ..... 25 cc.

Table 1. Age of Cultures of Anaerobic Bacilli When Stained for Flagella, Ordinarily Cultured on Blood Agar Slants Under Alkaline Pyrogallol, and Incubated at 37° C.

Culture No.	Name of species	Age of culture in hours when stained
79	*Bacillus Sordellii*	11
156	*Bacillus flabelliferum*	11
157	*Bacillus subterminalis*	15
63	*Bacillus tetani*	18
83	*Bacillus septicus*	18
158	*Bacillus amylobutyricus*	20
69	*Bacillus multifermentans*	20
88	*Bacillus botulinus, Type A*	22
99	*Bacillus Novyi*	22
12	*Bacillus putrificus*	24
95	*Bacillus Beijerinckii**	24
159	*Bacillus saccharobutyricus**	24
72	*Bacillus Pasteurianum**	24
2	*Bacillus difficilis*	36

*These three species were cultured on starch potato medium and incubated at 30° C.

The tannic acid solution was prepared as fresh as possible, because it was found that mold would grow in solutions left standing more than a week. The $FeCl_2$ solution was made up in larger amounts, as there was apparently no deterioration. The mordant was mixed just before use, and the $FeCl_2$ *added to* the tannic acid. This was filtered onto the slide to be stained, and left for 3 minutes. The mordant was not washed off. (If left on 4 minutes or longer more precipitate was formed; if left on less than 3 minutes staining was apt to be faint.)

[2]The following chemicals were employed: Tannic acid, C.P., Baker, Lot No. 21434; $FeCl_2 \cdot 6H_2O$, Baker, Lot No. 41636.

Staining fluid:

Mordant	0.7 cc.
Carbol Fuchsin, (Ziehl-Neelsen)[3]	0.1 cc.
HCl, C.P., concentrated	0.1 cc.
Formalin (40% formaldehyde)	0.4 cc.

The stain was mixed fresh each time just before using, adding the solutions in the given order. A small evaporating dish was used, and each solution rotated as it was added. This seemed to prevent a precipitate from forming. The above amount was enough to cover one 3 × 1 inch glass slide. The stain was filtered immediately onto the slide and left for 7 minutes, then washed off with distilled water (filtered thru a Mandler filter) blown gently thru a wash bottle. The slide was not allowed to dry.

Finally, the slide was covered with freshly filtered Ziehl-Neelsen's carbol fuchsin, prepared as described, and heated for one-half to one minute. It was found that careful experimentation was required as to the amount of heat needed. Too much heat would destroy the flagella and cause a great amount of precipitate; not enough heat would stain the flagella too faintly or not at all. The stain was poured off and washed with distilled water blown gently thru a wash bottle, as before. The slides were air-dried, and not blotted.

It was found from experience that by following this technic rather closely much better stains were produced and the results were more consistent. This is illustrated by the photomicrographs[4] in Figures 1 to 9 which were stained by the methods usually described (Bailey, 1929), and Figures 10 to 14 prepared by following very closely the technic outlined here.

After many unsuccessful attempts to obtain well-stained slides for photomicrographs of the flagella of *B. difficilis* included in the articles describing this organism (Hall and O'Toole, 1933; Snyder, 1937), it was decided to try the following experiment on the assumption that the flagella of freshly isolated pathogenic strains of anaerobic bacilli are more vigorous, and therefore more easily stained. This

---

[3]Carbol fuchsin was prepared as follows:

10% basic fuchsin in 95% ethyl alcohol (at least 20 hr. old)	10 cc.
5% aqueous phenol	90 cc.

Filtered, after mixing.

The following chemicals were employed: Phenol, C.P., Baker, Lot No. 42841; basic fuchsin, pure crystals, Coleman and Bell, Lot No. 390547 (not certified).

[4]Acknowledgment of appreciation is made to Mr. Glenn Mills, Department of Photography, University of Colorado School of Medicine and Hospitals, for the photomicrographs.

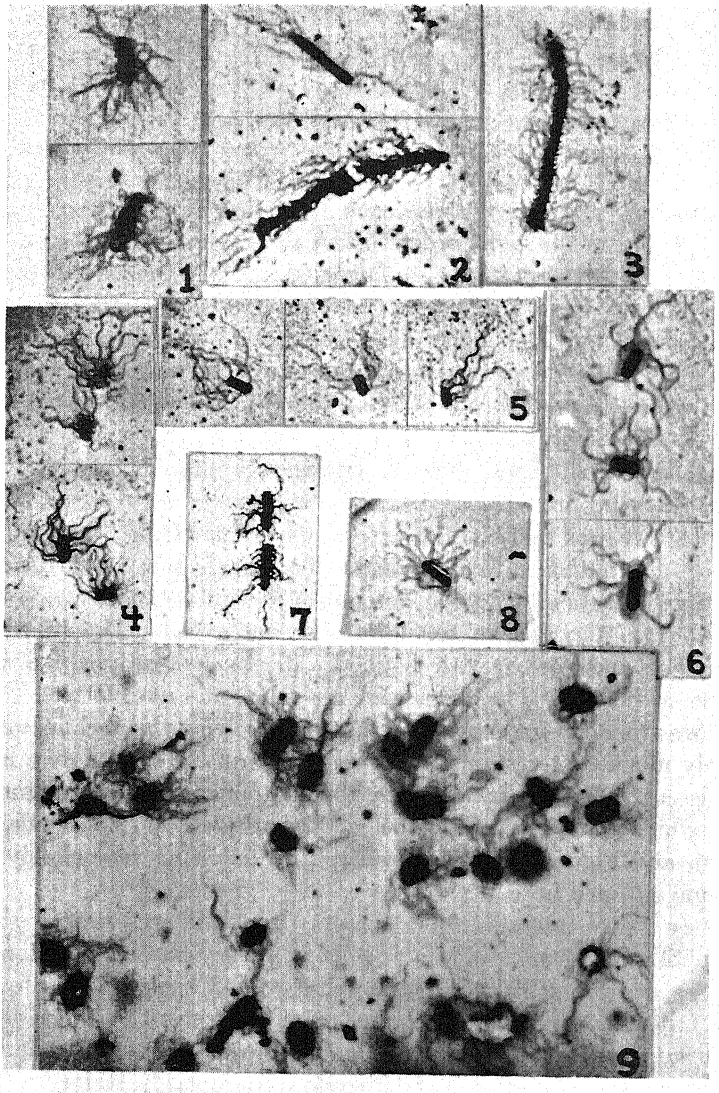


Plate I. Explanation of figures

Photomicrographs of flagella of anaerobic bacilli stained by Bailey's method. All cultures except *Bacillus Beijerinckii* were grown on blood agar slants under alkaline pyrogallol, and incubated at 37° C.; but that one (Fig. 6) was grown on potato agar slant and incubated at 30° C.

Fig. 1. *Bacillus Novyi.* 22 hour culture. × 1200.
Fig. 2. *Bacillus putrificus.* 24 hour culture. × 1000.
Fig. 3. *Bacillus amylobutyricus.* 20 hour culture. × 1000.
Fig. 4. *Bacillus flabelliferum.* 11 hour culture. × 1000.
Fig. 5. *Bacillus subterminalis.* 15 hour culture. × 1000.
Fig. 6. *Bacillus Beijerinckii.* 24 hour culture. × 1200.
Fig. 7. *Bacillus Sordellii.* 11 hour culture. × 1000.
Fig. 8. *Bacillus multifermentans.* 20 hour culture. × 1000.
Fig. 9. *Bacillus botulinus,* Type A. 22 hour culture. × 1200.

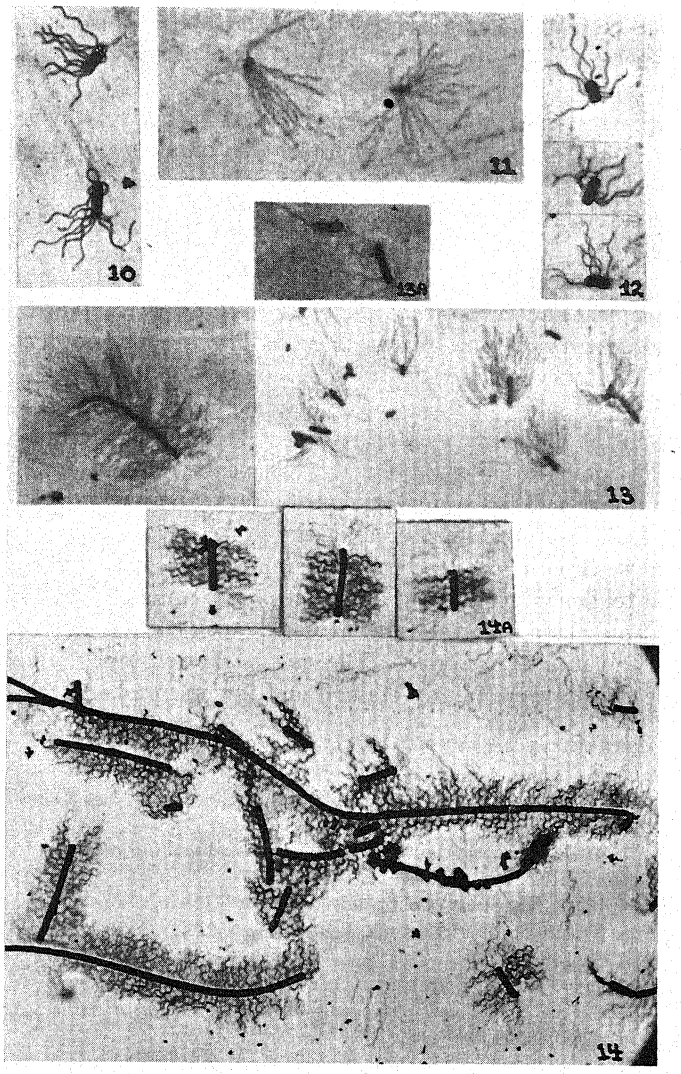


Plate II. Explanation of figures

Photomicrographs of flagella of anaerobic bacilli stained by Bailey's method. Three cultures (Figs. 11, 13, 14) were grown on blood agar slants under alkaline pyrogallol and incubated at 37° C.; the other two (Figs. 10 and 12) on potato agar slants and incubated at 30° C.

Fig. 10. *Bacillus saccharobutyricus.* 24 hour culture. × 950.
Fig. 11. *Bacillus septicus.* 18 hour culture. × 800.
Fig. 12. *Bacillus Pasteurianum.* 24 hour culture. × 950.
Figs. 13 and 13A. *Bacillus difficilis.* 36 hour culture. Results of staining from freshly isolated strain after guinea pig inoculation (13); same strain from three year old stock culture (13A). (13, × 800) (13A, × 950)
Figs. 14 and 14A. *Bacillus tetani.* 18 hour culture. × 800. Long filaments which extended for almost two microscopic fields (14); individual bacilli (14A).

seems to be borne out by the results obtained, at least for this particular species. The strain (No. 2) used had been sealed as a stock culture in deep brain medium for 3 years.

A guinea pig weighing 250 g. was injected with 2 cc. of the supernatant fluid from a 48 hour growth of this organism in deep brain medium. Within 48 hours the guinea pig was dead; autopsy revealed extended, subcutaneous edema which was very gelatinous and hemorrhagic. Cultures were made from this area, precautions being taken to avoid contamination. With appropriate and careful microscopical and cultural methods the bacillus corresponded to the original description, and proved to be pathogenic for another guinea pig with autopsy findings as described above.

Growth of this organism on blood agar slants under alkaline pyrogallol at 36 hours was faint but definite, and motility under coverslip preparation was sluggish. Smears stained for flagella by the above method were very satisfactory, the background being fairly free from precipitate, and the rods deeply stained. The flagella were peritrichous, very numerous, wavy, nicely spread out without tendency to tangle, and of more or less equal length. (Fig. 13).

A culture made from the three-year-old stock culture was used as a control and stained as above. The smears showed well stained rods, but the flagella were faintly stained and few in number. (Fig. 13A).

REFERENCES

BAILEY, H. D. 1929. A flagella and capsule stain for bacteria. Soc. Exp. Biol. and Med., 27, 111–2.

HALL, I. C. 1921. A constricted tube with mechanical seal for anaerobic fermentation tests. J. Inf. Dis., 29, 317–20.

HALL, I. C. 1932. The study of obligately anaerobic bacteria, *in* Manual of Methods for Pure Culture Study of Bacteria, Leaflet III, *2nd Ed.* Soc. of Amer. Bacteriologists, Geneva, N. Y.

HALL, I. C., and O'TOOLE, ELIZABETH. 1935. Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Amer. J. Diseases Children, 49, 390–402.

LEVENSON, SERGE. 1936. Coloration des cils bactériens par un procédé simple. Ann. Inst. Pasteur, 56, 634.

SNYDER, M. L. 1937. Further studies on *Bacillus difficilis* (Hall and O'Toole). J. Inf. Dis., 60, 223–31.

NOTES ON TECHNIC

Staining Scab Actinomyces in Aerial Potato Parts

In a recent paper, Hutchins and Lutman[1] described the staining of *Actinomyces* hyphae imbedded in the middle lamellae of potato tuber cells by use of a modified Gram's stain. The method was successful in staining the mycelium of the parasite in potato roots as well as in the tubers, but when an attempt was made to determine whether aerial portions of the plant were also infected, some further modifications had to be made.

In the method used for tubers, sections were washed in absolute alcohol until no more color flowed from the slide (30 to 60 min.) but when stem sections were tried, they were completely decolorized. To retain the stain, washing in absolute alcohol had to be limited to a few seconds, best results being obtained by merely flooding the slide with it once or twice, then placing in xylol for 24 hours. This prolonged immersion in xylol serves to prevent a later diffusion of crystal violet into the balsam after mounting and so renders the color in preparations more permanent. Differentiation continues very slowly in the xylol and would cause the loss of color in the preparations mounted in Canada balsam dissolved in this solvent. Filaments stained and observed in aerial organs of the potato plant were much finer than those seen in the tubers, and this fact probably accounts for the rapid decolorization of the sections in absolute alcohol which has made necessary a revision of the original technic.

Difficulty was encountered in the use of freshly made solutions of crystal violet, the first 20 to 40 slides being covered with what appeared to be an emulsion of minute droplets of anilin oil even tho the staining solution had been filtered very thoroly thru hard filter paper. These may be avoided by allowing the crystal violet solution to stand for 48 hours, then filtering and repeating the process.—H. E. Wheeler and B. F. Lutman, *Vermont Agricultural Experiment Station, Burlington, Vt.*

Iron-hematoxylin Staining of Salivary Gland Chromosomes in Drosophila

Salivary gland chromosomes, in the genus *Drosophila*, can be stained with iron hematoxylin and permanent mounts made of them

[1]Hutchins, H. L., and Lutman, B. F. Staining scab Actinomyces in potato tuber tissues. Stain Techn., **16**, 63–6. 1941.

by the technic which follows. Preparations put up in this manner have been kept for more than 2 years and are still in good condition.

The method used is a modification of Bauer's.[2] Salivary glands are fixed from 1 to 2 hours in 50% glacial acetic acid which has been prechilled. After fixing, the glands are smeared on albumen-coated slides.

Bauer's albumen solution is made by mixing 100 cc. distilled water, 25 g. powdered egg albumen (Merck), and 0.5 g. thymol. The mixture is allowed to stand overnight, until the excess of albumen has settled out. The supernatant liquid is decanted and diluted with distilled water to 200 cc., and preserved by 0.5 g. of thymol. When this solution is spread on the slides it produces an excessively heavy coat of albumen. Better results may be obtained by diluting the stock solution with water in the proportion of 1 cc. of Bauer's solution to 8 cc. of distilled water. Other dilutions have been tried, but this proves to give the best results. After being coated, the slides are allowed to become thoroly dry.

The fixed glands are placed on an albuminized slide in about 3 drops of fixative and carefully covered with a cover glass. The cover glass is then drawn across the slide, smearing the gland cells and drawing the chromosomes out of their coils into fairly straight figures.

The slides are next placed in Coplin jars of the fixative until the cover glass loosens and drops off. Then the slides are washed for 5 minutes under gently running tap-water, after which they are placed in a 5% $NaHCO_3$ solution for 5 to 10 minutes. Neutralizing the acid fixative at this stage causes the stain to act more satisfactorily. The slides should be washed thoroly, then mordanted in a freshly filtered 2% aqueous solution of ferric ammonium sulphate. After mordanting, the slides are washed for an hour in several changes of distilled water. They are then stained in 0.5% aqueous hematoxylin solution for 5 hours. After staining, the slides are washed, and differentiated with 0.2% aqueous ferric ammonium sulphate solution. When the preparations have been differentiated until the bands of the chromosomes are clearly visible, they are washed in numerous changes of distilled water or in running water, which should be neutral in reaction and free from iron salts. The preparations are then dehydrated, and mounted in damar or clarite.—LAWRENCE E. GRIFFIN and AGNES M. MCQUARRIE, *Reed College, Portland, Ore.*

[2]Bauer, Hans. 1936. Notes on permanent preparations of salivary gland chromosomes. Drosophila Information Service, 6, 35–6.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

GAGE, SIMON HENRY. **The Microscope.** 6 x 9 in. 617 pp. Cloth. 313 illustrations. Comstock Publishing Co., Ithaca, N. Y. 1941. $4.00.

It is very unusual for anyone to undertake personally the preparation of a 17th edition of a scientific treatise of which he was the original author. Just that, however, is what Dr. Gage has accomplished in making the latest revision of his well-known text. The nature of that revision can best be expressed by quoting the first two paragraphs of the preface to this new edition:

"In revising the matter for this, the seventeenth edition of *The Microscope*, changes have been made in every chapter in text and often in illustrations to render the subject more easily understood.

"Attention has been called to the newly devised Electron Microscope with its greatly increased magnifying power and resolution over the ordinary microscope; to Polaroid for the micro-polariscope; to some new plastics for mounting in place of Canada balsam; to the high-pressure mercury lamps for ultra-violet radiation and the bright mercury lines for photographing objects with the microscope."—*H. J. Conn.*

GUILLIERMOND, ALEXANDRE. **The Cytoplasm of the Plant Cell.** *Translated from the French manuscript by* Lenette R. Atkinson. 7 x 10¼ in. Cloth. 247 pp. 151 illustrations. Chronica Botanica Co., Waltham, Mass. 1941. $4.75.

The author of this book is such a well-recognized authority on the subject that the book's value and usefulness are beyond question. Most of the subject matter, however, lies outside the scope of these reviews, as it does not deal with dyes or with microtechnic. Its chief value is as a resumé of work on plant cytoplasm and the author's interpretation of the structures brought out by refined microtechnical methods. Especially interesting is his discussion of the chondriosomes, with its review of the literature on this subject, including the author's interpretation of the facts observed and his comments on the theories proposed.

Altho in such a subject as this, frequent reference to technic is naturally inevitable, the only chapter which gives much attention to this subject is one entitled "Vital Staining of the Vacuoles". This chapter is very interesting from the standpoint of anyone concerned with the physiological action of dyes and their ability to penetrate living cytoplasm. A study is made of three vital dyes and of the amount of each dye absorbed by living yeast cells at different pH-values. The amount of dye absorbed from the fluid is correlated with the speed with which the dyes appear in the vacuoles; and conclusions of much interest are drawn concerning the toxicity of the dyes and the ability of the cells to eliminate them.—*H. J. Conn.*

KOLMER, JOHN A. and BOERNER, FRED. **Approved Laboratory Technic.** *3rd Ed.* 6½ x 9½ in. 921 pp. Cloth. 380 illustrations. Index. D. Appleton-Century Co., New York City. 1941. $8.00.

This is the first edition of this book to be reviewed in these pages, altho previous editions have appeared in 1931 and 1938 respectively. The first edition of this book was stated on the title page to be "Prepared under the Auspices of the American Society of Clinical Pathologists." It bore, however, an introductory note explaining that the methods given therein for the diagnosis of disease were not approved by the Society; the approval extended merely to the technic employed in those methods. The preface, moreover, bore the statement that the

technic, in every instance, was approved by at least five members of the Society. The present edition of the book, on the other hand, avoids any chance of misunderstanding which might arise from these statements; because the name of the Society is omitted from the title page of the book, and the authors assume responsibility for the methods, in coöperation with a list of 28 collaborators.

Since the first edition, the book has increased considerably in length, but not in bulk, thanks to the use of thinner paper. It covers a very wide range of methods which are grouped in the following headings: General Laboratory Methods; Clinical Pathological Methods; Bacteriological Methods; Serological Methods; Chemical Methods.

Microtechnic is treated in various places thruout the book. The very first chapter of the book is given up to the microscope and methods of micrometry. One long chapter under Clinical Pathological Methods is given over to methods for the examination of blood, while other chapters following deal with the examination of other body fluids and exudates; altho many of the methods are chemical, microtechnic is naturally included. The bacteriological section, in the same way, naturally includes much microtechnic; while the final chapter of the book deals with histological methods and the preparation of museum specimens.

The present edition of the book should be useful, not only to clinical technicians, but to laboratory workers in others branches of Bacteriology.—*H. J. Conn.*

MICROSCOPE AND OTHER APPARATUS

REES, A. L. G. **The electron microscope.** *Chem. and Ind.*, **60**, 335. 1941.

This paper is a technical discussion of the development of optical microscopy and of the different types of electron microscopes. For purposes of comparison the resolutions of which the various microscopes are capable are tabulated alongside certain familiar dimensions; for example: bacteria and colloid particles require either oil immersion microscope, scanning electron microscope or quartz-u. v. microscope; viruses require shadow electron or electrostatic electron microscopes; and protein molecules require the magnetic electron microscope. Of particular interest to biologists, many examples of new structural details of bacteria and viruses exposed by the electron optical method are cited, and mention is made of its application to the study of cell tissues. The author stresses the great possibilities of electron microscopy, the necessity for improvements and the opportunity for development in this line of work.—*A. P. Bradshaw.*

MICROTECHNIC IN GENERAL

HERMAN, CARTON M. **Isopropyl alcohol as a dehydrating agent and preservative for biological specimens.** *J. Lab. & Clin. Med.*, **26**, 1788. 1941.

Isopropyl alcohol can be used instead of ethyl alcohol as a solvent for stains, as a dehydrating agent, and as a preservative for biological material. The method is inexpensive and satisfactory.—*John T. Myers.*

LIESEGANG, R. E. **Eine Hemmung der Formalinfixierung.** *Zts. wiss. Mikr.*, **57**, 162–3. 1940.

The author investigated disturbances in formalin fixation of histological preparations which contained variable quantities of urea. Experiments carried out on the coagulation of gelatin by means of formalin in the presence of urea showed that the degree of coagulation was lowered materially. It was thought that the cause rested in the breaking up of the molecules by the urea, for it is well known that technical gelatin always contains portions of small molecular gelatose which in contrast to the glutin molecules are not coagulated by formalin. Even a 40% gelatin solution remained liquified in the cold if it contained sufficient urea; however, this urea content was entirely too low to bring about very extensive molecular splitting. Another cause had to be sought; this was found on mixing equal quantities of formalin and concentrated urea in the absence of gelatin. After a few hours a white turbidity became manifest. Later the entire

solution congealed to a solid mass. Thus a condensation product was formed conforming to the many products of similar nature known in the chemistry of plastics. The action of formalin on gelatin was inhibited, as was also the effect of urea. If a small quantity of any acid was added to the freshly prepared mixture, turbidity and coagulation occurred in a few seconds. This technical step is also familiar in the manufacture of synthetic resins. It was unknown, however, that the formation of a synthetic resin thru inactivation of the formalin was somewhat delayed but not permanently inhibited in the presence of gelatin.—*J. M. Thuringer.*

DYES AND THEIR BIOLOGICAL USES

ALBERT, ADRIAN, and RITCHIE, BRUCE. **Improved syntheses of aminoacridines. Part I. The five isomeric monoaminoacridines.** *J. Soc. Chem. Ind.*, **60**, 120. 1941.

Altho a number of useful antiseptics and dyestuffs are derived from diaminoacridines, the monoaminoacridines have received scant attention because of their comparative inaccessibility. These monoamines have been prepared conveniently and in good yield by modifications of methods given in the literature, several unnecessary stages being eliminated. A new source of 2-aminoacridine was found in the readily accessible 3′-nitrodiphenylamine-2-carboxylic-acid, since contrary to published statements, the direction of ring closure can be controlled to give either (mainly) 2- or 4-aminoacridine.

Two of these isomerides possess strong antiseptic powers, 2-aminoacridine inhibiting streptococci at a dilution of 1 in 40,000 and 5-aminoacridine at 1 in 80,000. The latter is unique among the acridine antiseptics in that it does not stain the skin. Two other dyes of this group give tannin-mordanted viscose a rose and a violet color respectively, colors not previously recorded for aminoacridine dyes.—*A. P. Bradshaw.*

BERGMANN, E., HASKELBERG, L., and BERGMANN, F. **Synthesis of lipophilic chemotherapeuticals. VI. Lipophilic substitutions in azo-dyes.** *J. Amer. Chem. Soc.*, **63**, 2243. 1941.

Fifteen acyl derivatives of 4-(benzene-azo)-naphthylamine-1 have been described. As the *N*-trichloro-acetyl derivative showed a slight curative effect on tuberculosis and leprosy, its molecular structure has been systematically varied and the activity of the new products tested. For this purpose 24 new acyl derivatives of 12 azo dyes have been prepared. (A description of the related chemotherapeutical experiments will be given elsewhere.)—*A. P. Bradshaw.*

LAW, R. S. **Chlorothymols. Some aspects of their bacteriostatic and fungistatic properties.** *J. Soc. Chem. Ind.*, **60**, 66. 1941.

Monochlorothymol and monochloroisothymol have been found effective in preserving casein, gelatin, glues and starches. The two forms of chlorothymol give similar results, except that the solid monochlorothymol, setting point 59–61°, is a little more effective in preserving starch mucilages than the liquid monochloroisothymol.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

BONDI, AMEDEO, Jr. **A rapid method for staining blood smears in determining opsonocytophagic indices.** *J. Lab. & Clin. Med.*, **26**, 1811. 1941.

Blood smears are stained as follows: Mix citrated blood with a saline suspension of *Brucella* cells and incubate 30 min. at 37° C. Prepare smears and dry quickly. Pass thru a Bunsen flame 4 times and allow to cool. Cover with Ziehl-Neelsen carbol fuchsin (diluted 1: 10 with water) for 2 min. Wash with tap water and allow to dry. Cells of *Brucella* and the nuclei of phagocytes stain a deep red. The cytoplasm is a light pink and granules do not stain. Red blood cells stain weakly. Phagocytosed bacteria are easily counted.—*John T. Myers.*

GORDON, HAROLD. **A method for preparing smears and sections of aspirated sternal marrow.** *J. Lab. & Clin. Med.*, **26**, 1784–8. 1941.

The technic is as follows: File thru an 18-gauge spinal puncture needle, leaving 1.5 in. attached to the shaft, and rebevel its point. Make a mark with a file 0.6 in. from the top, to serve as a guide to the depth of penetration. Cleanse the midline of the sternum with iodine and alcohol, insert the needle to the mark and remove the stylet. Attach a syringe and aspirate the marrow. Smear, dry, and stain by Wright's method. Implantation metastases are avoided by this method. —*John T. Myers.*

ROSEN, THEODORE S., and LUBINSKY, GEORGE W. **Freezing of tissues with "dry ice" for microtome sectioning of the entire brain.** *J. Lab. & Clin. Med.*, **26**, 1799–1802. 1941.

The freezing attachment for the microtome is made as follows: Secure a piece of sheet aluminum $\frac{1}{8} \times 6\frac{1}{2} \times 5$ in. and screw it to a bakelite box measuring $6\frac{13}{16} \times 5\frac{5}{16} \times 2\frac{5}{16}$ in., of which it forms the roof. Cut away the end of the box and insert a wooden drawer built to fit into the box. The drawer contains a slab of dry ice which is forced against the aluminum plate by a false bottom mounted on spiral springs. The springs slide on thin metal tubes each of which surrounds a bolt. A washer and nut limit the upper excursion of the false bottom. The bakelite box is screwed to the object clamp supporting the base of the microtome. The method is inexpensive. The apparatus requires little space and is easily transported.—*John T. Myers.*

RUSSELL, W. O. **The substitution of zinc chloride for mercuric chloride in Zenker's fluid.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **21**, 47. 1941.

Because of the expense of $HgCl_2$ and the difficulty in obtaining it, a search was made for a satisfactory substitute to use in Zenker's fluid. $CaCl_2$ and $ZnCl_2$ were found to give as good staining results as $HgCl_2$ except in the phloxine-methylene-blue stain. These compounds were completely removed from sections by the usual dehydrating procedures, so that extra steps, like those required to remove excess crystals of $HgCl_2$, were unnecessary. $ZnCl_2$ is recommended because of cheapness since its cost in Zenker's fluid is only 1/10 that of $HgCl_2$.—*Jean E. Conn.*

SHOHL, ALFRED T., and HUNTER, THOMAS H. **The measurement of cell volume of blood by the Evans blue dye method.** *J. Lab. & Clin. Med.*, **26**, 1829–37. 1941.

The procedure used is as follows: Dry Evans blue (Merck) at 100°C. and dissolve in distilled water so that 1 liter contains 400–800 mg. Place 0.5–1.0 cc. of this in a tube of 3–4 cc. capacity and evaporate to dryness at 70°C. Collect blood so that it contains 0.2% of ammonium oxalate or 2.0–2.5 units of heparin per cc. Centrifuge and add 1.0 cc. of plasma to the dye tube. Remove 0.1 cc. of the dye-plasma to 9.9 cc. of saline in a photo-electric colorimeter tube. Prepare a blank with plasma omitted. Compare in a suitable colorimeter, as the Evelyn or Klet-Summerson, arranged to use a filter which transmits nothing but light at approximately 620mμ. Calculate according to the directions for the particular colorimeter.—*John T. Myers.*

PLANT MICROTECHNIC

KLEIN, RICHARD. **A new method of mounting expanded and unexpanded pollen grains.** *Bull. Amer. Soc. Amateur Microscopists*, **1**, 11. 1941.

For mounting expanded grains a drop of molten glycerin jelly (Brant's) is placed on a slide, allowed to harden, and covered lightly. When a specimen is obtained, the jelly is melted by a match flame and the cover glass is touched to the anther. After the cover is replaced the slide can be examined. To stain the pollen, methyl green or basic fuchsin is added to the jelly; see Wodehouse (Pollen Grains. McGraw Hill, N. Y. 1935. p. 106).

To mount unexpanded grains, white petroleum jelly (vaseline) can be used. The same technic is followed, but the vaseline does not have to be remelted before

touching the anther. To stain unexpanded grains, 2 parts of stain (1% aqueous) plus 1 part of absolute amyl alcohol are added to 8 parts of the vaseline. The alcohol acts as a wetting agent.

A neater looking mount may be made if the preparation is warmed to distribute the excess stain. Ringing any of these preparations will make them permanent. A dilute solution of Canada Balsam in xylol is effective.

This general technic has the following advantages: slides may be prepared in quantity in advance; the flowers will not be injured; there is little chance of contamination by foreign pollen or debris; very little manipulation is required. —*Author abstract.*

MICROÖRGANISMS

MATTICK, A. T. R., SHATTOCK, P. M. F., and JACOB, M. M. **The relationship of methods of bacteriological examination to the eradication and control of mastitis. I. The use of an enrichment technique in revealing streptococcal infections of the cow's udder. II. Streptococcus agalactiae infections in heifers.** *J. Dairy Research*, 12, 139–54. 1941.

An enrichment procedure is proposed which consists of a modified Hotis test in which 9.5 ml. of milk is added to 0.5 ml. of brom cresol purple in dilute ethyl alcohol (strength not stated) to give a final dye concentration of 0.025%; incubation is at 37°C. for 24 hr., followed by streaking on Edward's aesculin crystal violet blood agar. The procedure was found to give positive tests for mastitis streptococci in numerous instances where use of (1) Edward's agar medium alone, (2) Edward's sodium azide broth procedure, (3) streaking material from azide broth tubes showing cloudiness or sediment onto aesculin crystal violet blood agar plates or (4) the Hotis test alone failed to detect such organisms. The Hotis test was not considered reliable as a specific test for *S. agalactiae* in milk. In some instances repeated use, at intervals, of the enrichment procedure was necessary in the case of those quarters from which only very small numbers of streptococci were being eliminated. Repeated and frequent use of other tests for mastitis streptococci was found to reduce the number of cases detectable only by the enrichment procedure. By using the enrichment procedure the possibility of calfhood infection was shown by the 4.5% incidence of *S. agalactiae* infection among heifers just freshened and still isolated from the milking herd and the 21% infection of first-lactation heifers in herds apparently free of mastitis streptococci detectable by the Edward's agar procedure.—*F. E. Nelson.*

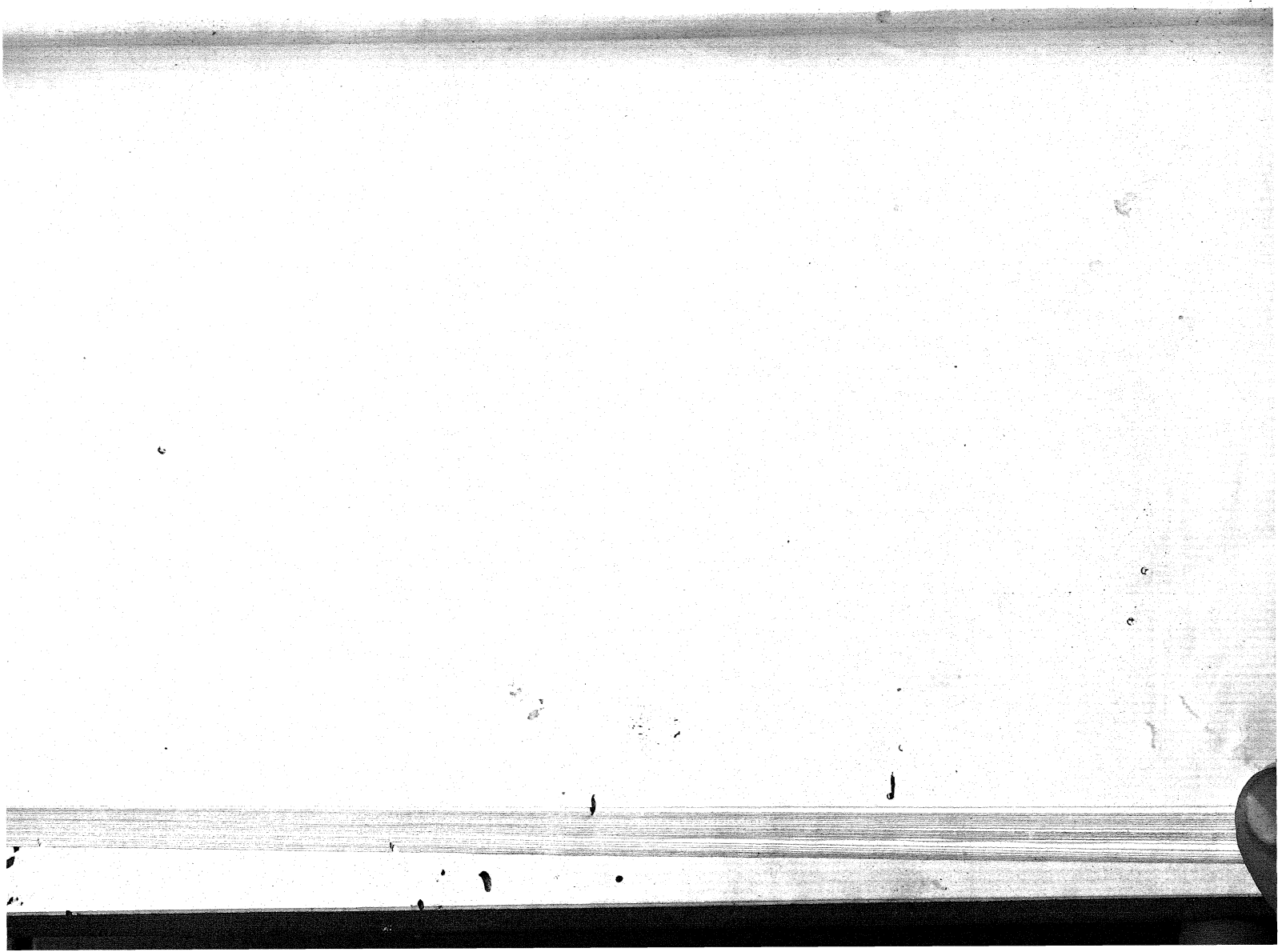
HISTOCHEMISTRY

STEIGER, ALF. **Mikrochemischer Nachweis des Karotin.** *Mikrokosmos*, 8, 121–2. 1941.

Carotin is found in crystalline form within the cells of many plants, notably roots such as the carrot. In thin razor sections of carrots mounted in water, carotin appears as yellow to orange crystals which may be rhomboidal, rectangular, needles or bundles of needles, also as spiny forms.

To demonstrate carotin in leaves and other green parts of plants, it is necessary to remove the chlorophyll and convert the carotin in solution into the crystalline form. Procedure: Place fresh leaves, stems, or sections of tissue into a mixture of saturated aqueous solution of KOH 1 part, 40% ethyl alcohol 2 parts, tap water 3 parts, (Molisch potash reagent). Use wide mouthed bottles with ground glass stopper or stender dishes with vaseline-sealed covers to prevent absorption of CO_2 from the air. Keep in the dark from one to several days, or until plant material is yellow and the solution is green. Transfer portions of the material to distilled water for several hours. Remove a small piece of material from the distilled water, dry with blotting paper and place on a slide. With a fine pipette drop a little concentrated H_2SO_4 on the material which at once turns dark green, then blue.

Under the microscope the cells are freed of their chlorophyll and the carotin crystals appear dark blue. A little time may elapse before the H_2SO_4 has a chance to penetrate everywhere; therefore, the crystals may not appear of uniform color. Occasionally carotin may at first not be clearly visible because the color of the crystals may be similar to that of the preparation.—*J. M. Thuringer.*



STAIN TECHNOLOGY

VOLUME 17 APRIL, 1942 NUMBER 2

ON THE STRUCTURE AND STAINING OF STARCH GRAINS OF THE POTATO TUBER[1]

JAMES C. BATES, *Kansas State College of Agriculture and Applied Science, Manhattan, Kansas*

ABSTRACT.—Procedures are described for the differential staining of starch grains of the potato tuber with hematoxylin, and for double staining with safranin 0 and fast green FCF. The staining effects obtained are made possible by the action of a swelling agent. Staining with hematoxylin is preceded by the swelling action of formaldehyde. In staining with safranin 0 and fast green FCF, the formaldehyde is added to the staining solution. The results obtained are as follows: (1) a clavate-shaped, central structure composed of small particles arranged in definite layers is revealed within the grain; (2) differential staining of the locus of the grain and the lamellae alternating with it in a small region around the longitudinal axis of the grain; (3) the simultaneous staining and separation of the grain into a cone-shaped peripheral portion and a spherical body containing the locus of the grain which emerges from it; and (4) differential staining of a ring or layer of substance around a spherical refractive body within the grain.

When starch grains from the potato tuber are mounted in water and examined under the microscope, one can observe the locus of the grain around which numerous eccentric layers of starch are formed (Fig. 1).

Some selective staining of the locus of the grain and alternate lamellae may be obtained by the following procedure:

1. Smear commercial potato starch on a slide previously treated with Szombathy's[2] gelatin adhesive, as modified by Haupt[3] and Adams[4].

[1]Contribution No. 423 from the Department of Botany and Plant Pathology, Kansas State College of Agriculture and Applied Science.

[2]Szombathy, K. 1918. Neue Methode zum Aufklefen von Paraffinschnitten. Zts. Wis. Mikr., 34, 334–6.

[3]Haupt, A. W. 1930. A gelatin fixative for paraffin sections. Stain Techn., 5, 97–8.

[4]Adams, J. E. 1940. A procedure for staining filamentous algae and fungi on the slide. Stain Techn., 15, 15.

STARCH GRAINS OF THE POTATO TUBER

FIG. 1. Eccentric striations of the grain.

FIGS. 2 and 19. Differential staining of the locus and alternate lamellae in a small region around the longitudinal axis of the grain.

FIG. 3. Clavate-shaped central structure unstained or slightly stained with hematoxylin.

FIG. 4. Clavate-shaped central structure showing numerous tiny granules which stain blue with hematoxylin.

FIGS. 5 and 20. Clavate-shaped central structure showing numerous brown-black appearing granules arranged in definite layers after staining with hematoxylin.

FIG. 6. Clavate-shaped central structure showing arrangement of needle-like crystals.

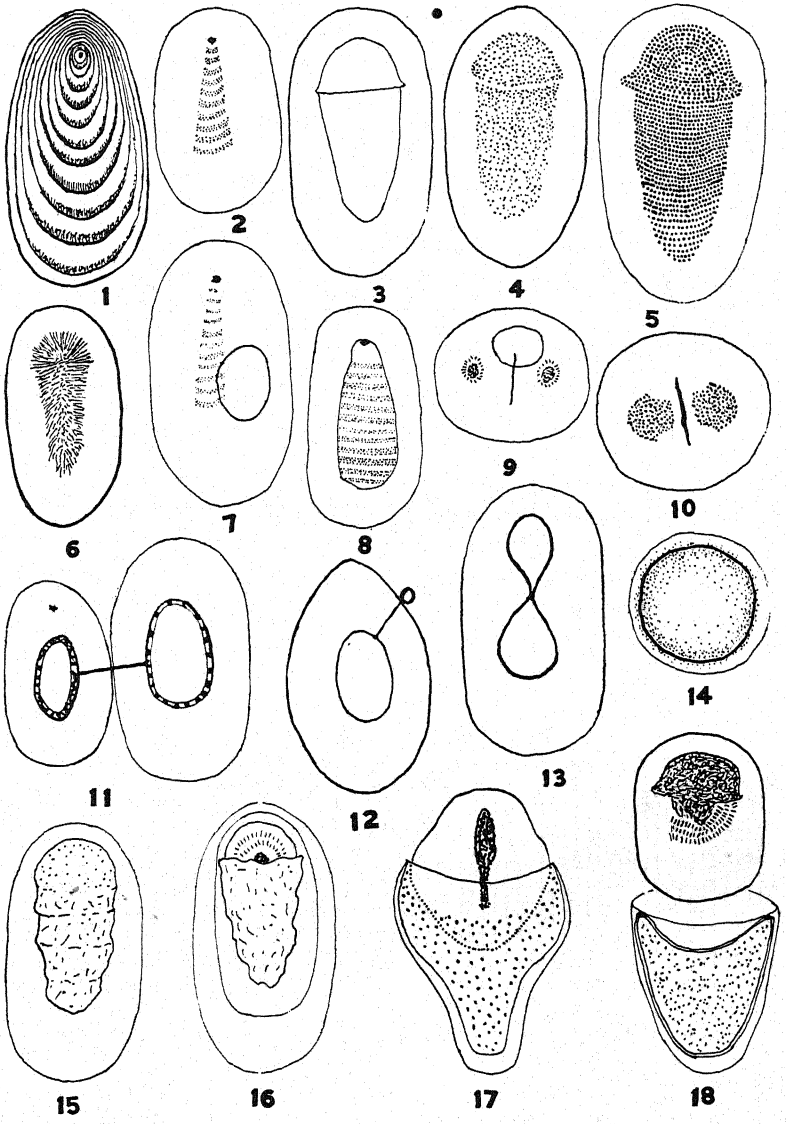
FIGS. 7, 8, and 9. Showing location of spherical refractive body within the starch grain.

FIG. 10. Showing clavate-shaped central structure in a compound grain.

FIG. 11. Reticulate ring of substance around the spherical refractive body which stains deep blue with hematoxylin.

FIGS. 12, 13, and 14. Showing layer of substance around the spherical refractive body which stains green with fast green FCF while the remainder of the grain stains red with safranin O. On standing the green color spreads until the red is completely obscured, Fig. 14.

FIGS. 15–18. Showing differential staining and stages in the separation of the grain into two parts.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

2. Treat 10 minutes with a 3% solution of commercial formaldehyde.

3. Expose 3 minutes to the fumes of commercial formaldehyde in a closed container.

4. Stain 30 minutes in the following solution of hematoxylin: 95% alcohol, 79 cc.; distilled water, 10 cc.; a 2% solution of hematoxylin (certification No. FH-10 used by author) in absolute alcohol, 5 cc.; a 2% aqueous solution of aqueous ferric chloride, 5 cc.; normal HCl, 1.3 cc.

5. Rinse in tap water, 10 minutes.

6. Dehydrate with alcohol. Clear in xylene. Mount in balsam.

In these preparations the locus of the grain and alternate lamellae in a small region around the longitudinal axis of the grain become rather conspicuous (Figs. 2 and 19). If the time of exposure to the fumes of commercial formaldehyde is increased from 3 to 30 minutes, the small particles composing the locus and alternate lamellae form needle-shaped crystals arranged at right angles to the lamellae (Fig. 6).

Considerable optical differentiation of the central and outer portions of the potato starch grain may be obtained by the following procedure:

1. Fix small pieces of recently dug potato tubers in CRAF killing fluid (1% chromic acid, 50 cc.; 10% acetic acid, 35 cc.; formaldehyde, 37–40% aqueous, 15 cc.), 24 to 48 hours.

2. Wash in running water.

3. Treat for 12 hours in each of the following: dioxan and water, 1:2; dioxan and water, 2:1; and 3 changes of pure dioxan.

4. Infiltrate with paraffin.

5. Section and flatten the paraffin ribbon by floating it on warm water at 35–40° C.

6. Mount the paraffin sections on a slide previously treated with Szombathy's gelatin adhesive, as modified by Haupt (*loc. cit.*).

7. Expose the sections to the fumes of commercial formaldehyde in a closed container, 36 hours.

8. Remove slides from the closed container and permit them to dry.

9. Dissolve off the paraffin with xylene, pass into 95% alcohol, then stain in the hematoxylin solution described above, 12–24 hours.

10. Rinse in tap water, dehydrate with alcohol, clear in xylene and mount in balsam.

The starch grain should show an inner clavate structure, dome-shaped at locus end, which remains unstained or only lightly stained

while the surrounding portion of the grain stains a light blue. (Fig. 3.) If the time of exposure to the fumes of formaldehyde is increased, numerous blue-staining granules will appear in the clavate structure (Fig. 4). If the time of exposure to the fumes of formalde-

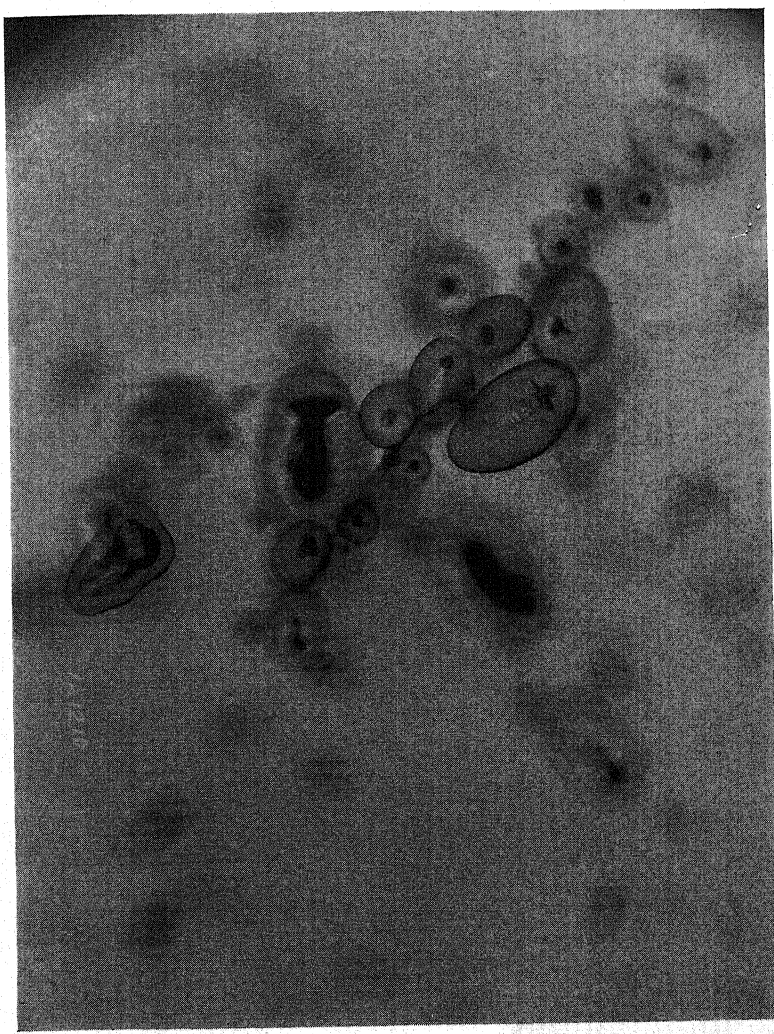


FIG. 19. Photomicrograph of starch grains showing the structure sketched in Fig. 2.

hyde is further increased, the blue-staining granules of the clavate structure will appear brown or black and one can observe that they are arranged in definite layers as shown in Figs. 5, 10, and 20. These brown-black staining particles appear under high magnification to

be in the form of tiny crystals. The dome-shaped end of the clavate structure appears to be the enlarged locus of the grain. If the time of exposure to the fumes of formaldehyde is further increased, the clavate structure will disintegrate leaving the brown-black staining particles in irregular arrangement.

The central and outer portions of the starch grain may be stained and simultaneously caused to separate by the following procedure:

1. Place commercial potato starch grains for 27 hours in a staining solution with formalin: commercial formaldehyde, 5 cc.; distilled water, 5 cc.; 65% ethyl alcohol, 2 cc.; safranin 0 (certification No. NS-10 used, dye content 94%), 10 mg.; fast green FCF (certification No. NGf-4 used, dye content 91%), 10 mg.; normal HCl, 0.04 cc.
2. Decant staining solution.
3. Rinse with water.
4. Dehydrate with alcohol. Clear in xylene. Mount in clarite.

During this treatment there will be considerable swelling of the grain and some staining. In some grains the clavate-shaped central portion becomes quite conspicuous, slightly granular, and swollen especially at the locus (Fig. 15). In some of the grains in which the swelling has progressed further, the membrane formed around the grain becomes thinner at the locus end and thicker at the opposite end and stains uniformly red. A layer enclosing the clavate structure dissolves or ruptures and exposes a spherical body containing the locus (Fig. 16). In other grains in which swelling has progressed further, the outer membrane formed around the grain ruptures at the locus end, and the spherical body containing the locus emerges and completely separates from the cone-shaped peripheral part of the grain (Figs. 17 and 18).

When potato starch grains are permitted to float on the surface of water and are examined under the microscope, one can observe within each grain one or sometimes two spherical-elliptical refractive bodies. The size and location of these refractive bodies may vary considerably. Sometimes they are located to one side of the longitudinal axis (Fig. 7), but more frequently are located as shown in Fig. 8. Fig. 9 shows the location of one in a compound grain.

Differential staining of a ring or layer of substance surrounding the refractive body may be obtained by the following procedure:

1. Smear commercial potato starch grains on a slide previously treated with Adam's gelatin adhesive.
2. Treat with a 3% solution of commercial formaldehyde for 10 minutes.
3. Expose to the fumes of commercial formaldehyde, 8 minutes.

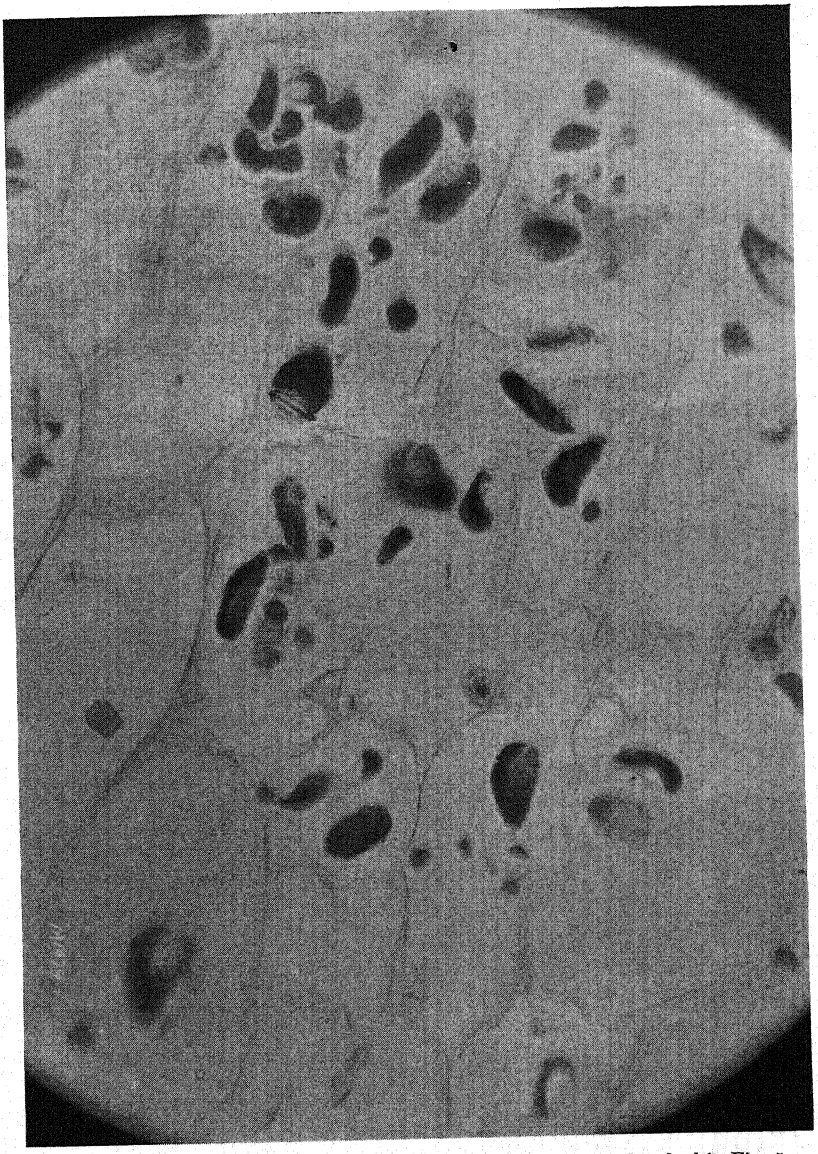


FIG. 20. Photomicrograph of starch grains showing structure sketched in Fig. 5.

4. Stain in the hematoxylin solution described above for 24 hours.

5. Rinse with tap water. Dehydrate with alcohol. Clear in xylene. Mount in balsam.

The starch grain will stain light blue, while a conspicuous deep blue ring will appear around the refractive body. Frequently when two starch grains come in contact with each other, a small strand of the deep blue staining material of the ring will pass out thru the peripheral portion of the grain and join with a similar strand passing out from the deep-blue-staining ring in an adjoining grain. The ring of deep-blue-staining material appears to be reticulated (Fig. 11).

If the staining solution described above, containing safranin 0 and fast green FCF, is used instead of the hematoxylin solution, the ring or layer of deep-blue-staining material around the refractive body stains green while the remainder of the grain stains red. If the action of the stain is observed under the microscope, one notes that the green ring is formed very quickly, continues to deepen, finally diffuses into the refractive body and out toward the surface until nearly all of the red color is obscured by the green. (Fig. 14.) Sometimes the green-staining material is forced out thru the peripheral portion in small strands and collects on the outside of the grain in small droplets (Fig. 12).

STUDIES ON POLYCHROME METHYLENE BLUE

I. Eosinates, their Spectra and Staining Capacity

R. D. LILLIE, *Senior Surgeon*, AND M. A. ROE, *Surgeon, USPHS*
Divisions of Pathology and of Infectious Diseases,
National Institute of Health, Bethesda, Maryland

ABSTRACT.—**The absorption spectra of eosinates of thiazin dyes in water exhibit absorption maxima at the same spectral locations as do the individual component dyes in aqueous solution.**

Commercial samples of Wright's stain showing thiazin absorption maxima between 620 and 660 mμ generally give satisfactory blood stains. Nuclear staining is redder and cytoplasm grayer blue in 620-640 range, and consequently staining of malaria parasites is less satisfactory in that range. The best malaria stains show their thiazin absorption maxima usually between 650 and 660 mμ.

Successive batches of Wright's stain made by the same manufacturer, as well as experimental laboratory lots, may show wide variations in their thiazin absorption maxima and in their staining characteristics.

Hematologists and malariologists have long been concerned with the variability in staining capacity for blood and for malaria parasites of various lots of the eosinates of polychrome methylene blue prepared by the Wright, Leishman, and other methods.

Recently in connection with published studies on the use of the various azures[1] and of their eosinates[2], we made extensive use of the spectrophotometer for the identification of the various methylene blue and azure samples of domestic and foreign manufacture. This method was used also by Holmes and co-workers[3], by Formanek[4] as well as Scott and French[5] on supposedly purified azures.

[1]Roe, M. A., Lillie, R. D., and Wilcox, A. 1940. American azures in the preparation of satisfactory Giemsa stains for malaria parasites. Pub. Health Rep., **55**, 1272–8. 1941.

[2]Roe, M. A., Wilcox, A., and Lillie, R. D. 1941. Eosinates of the azures and methylene blue in preparation of a satisfactory Giemsa stain from dyes of American manufacture. Pub. Health Rep., **56**, 1906–9.

[3]Holmes, W. C. 1928. The spectrophotometric evaluation of mixtures of methylene blue and trimethyl thionin. Stain Techn., **3**, 45–8.

Holmes, W. C., and Snyder, E. F. 1929. The atmospheric oxidation, or dealkylation, of aqueous solutions of methylene blue. Stain Techn., **4**, 7–10.

[4]Formanek. 1908. Untersuchung und Nachweis organischer Farbstoffe. Berlin. Cited from Scott and French.

[5]Scott, R. E., and French, R. W. 1924. Standardization of biological stains. Mil. Surg., **55**, 229–43 and 337–52.

It does not, however, seem to have been applied to the study of the precipitated and dried eosinates. Preliminary trials showed that a solution of Wright's stain (NWr-10) in C. P. methanol diluted with the same solvent about 1,000 times gave a clearly defined absorption spectrum with absorption maxima at 525 and 648–650 mμ. An unpolychromed methylene blue eosinate diluted in the same way in C. P. methanol gave maxima at 525 and 652 mμ, and diluted in 95% ethyl alcohol at 528–530 and 654 mμ. As the parent lots of eosin Y (LE-11) and methylene blue (E&A old) gave maxima in 95% alcohol at 531 and 656 mμ respectively, this method of exploration was thought worthy of further trial.

Next it was found that dilutions of methanol and methanol-glycerol solutions of thiazin eosinates in distilled water to give final dilutions between 5 and 10 γ per cc. were sufficiently stable to give quite constant readings on the spectrophotometer at any given wave-length over a period of several hours.

TABLE 1. ABSORPTION MAXIMA OF THIAZINS AND THEIR EOSINATES IN WATER*

Graph	Dye and Lot Number	Absorption Maxima in mμ
1	Methylene blue, E. & A., Lot I-K	664
63	Eosin Y, LE-11	517
68	Methylene blue eosinate, Lot I-K (E. & A.+LE-11)	517, 663
72	Azure A, NAz-6	618–620
70	Azure A eosinate (NAz-6+LE-11)	517, 612–622
73	Azure B, NAC No. 9348	652
71	Azure B eosinate (NAC No. 9348+LE-11)	517, 653
85	Polychrome methylene blue, Lot IV EA	652
90	Polychrome methylene blue eosinate (Lot IV EA+LE-11)	517, 652
87	Polychrome methylene blue, Lot IV EB	652–658
91	Polychrome methylene blue eosinate (Lot IV EB+LE-11)	517, 652–660
22	Polychrome methylene blue, Lot IV B	653–658
92	Polychrome methylene blue eosinate (Lot IV B+LE-11)	517, 652–657

*Eosinates were dissolved 0.1 g. to 80 cc. C. P. methanol and diluted with distilled water to give 25 to 30% transmittance at 517 mμ on the Coleman spectrophotometer, usually about 1:200. Readings were taken at 10 mμ intervals from 400 to 800 mμ, at 2 mμ intervals for 10–15 mμ to each side of the maxima and at 5 mμ intervals thru the remainder of the 610 to 690 mμ band.

The results of spectrophotometric examination of several lots of methylene blue, of polychrome methylene blue, and of the various azures are presented in Table 1, together with similar data for the eosinates prepared from the same dye samples with the same sample of eosin. Apparently the absorption spectrum of an eosinate in water presents the same absorption maxima as its component dyes do separately in aqueous solution.

Thin blood films from patients heavily infected with *Plasmodium malariae* were prepared and stained with 26 samples of various

Romanowsky stains (0.1 g. to 80 cc. of C. P. methanol), using thruout prior fixation in C. P. methanol at the bedside and staining not over 24 hours later. Undiluted stain (1 cc.) was applied for 1 minute, then 2 cc. of water buffered to pH 6.5 or to pH 7.0 was added and staining continued for 5 and for 10 minutes.

General ratings were made of these films for the effectiveness of the stain for blood morphology and for parasites. In general it was noted that the longer staining interval and the higher pH-level gave denser nuclear staining, heavier neutrophil granules and denser parasite chromatin. The general effects of each stain are tabulated in Table 2 together with spectrophotometric data on the corresponding samples.

Besides the absorption maxima for the eosin and for the thiazin dyes concerned we have included the width and median point of the band in which D, the logarithm of the reciprocal of the transmittance percentage (T), is at or over 90% of its maximum for the thiazin dye. The purpose of the introduction of these latter is to differentiate between samples showing relatively complex polychrome methylene blues and consequently broad absorption bands, and relatively pure thiazins with narrower bands.

In general it is seen that fairly good blood stains were obtained with most samples showing thiazin absorption maxima between 620 and 660 mμ. Stains with maxima above 660 tend to show irregularity of leucocyte nuclear staining, some cells showing the pale blue of methylene blue eosinate stains. Satisfactory staining of plasmodial chromatin and cytoplasm was restricted to a narrower band, between 650 and 660 mμ. The one sample (Balch stain) with a much lower maximum (624 mμ) which gave good parasite staining had a quite wide absorption band whose median was nearly 10 mμ higher than the recorded maximum. This indicates a considerable mixture of azure B (absorption maximum 652) with a predominant azure A (absorption maximum about 620–624).

These findings agree well with the results of previous studies of the azures and their eosinates in relation to malaria parasite staining. (See footnotes 1 and 2.)

It is noteworthy that 3 successive samples of Wright's stains from the same manufacturer showed quite divergent thiazin absorption maxima and staining characteristics: NWr-13, 656 mμ; NWr-14, 627 mμ; and NWr-15, 660 mμ; and similarly, CWr-12 at 660 mμ and CWr-13 at 620 mμ for another manufacturer. Note should also be made in passing of the discordant results with the year-old and fresh solutions of NWr-13, with maxima respectively at 607 and 656 mμ.

TABLE 2. SPECTROPHOTOMETRIC DATA AND STAINING PERFORMANCE OF COMMERCIAL SAMPLES OF ROMANOWSKY STAINS

Graph No.	Stain and Lot	Absorption maxima		Wave-length band of 90% D λ*			Staining performance†	
		Fluorane	Thiazin	Range λ	Width	Median λ	Blood	Paras.
139	Thionin Eosinate HL38, 12–18–40	516	600	587.0–606.9	19.9	596.9	±	±
120	Wr. Biosol., 1935 sol.	515	605	589.9–630.9	41.0	610.4	±	±
126	NWr-13, 11–24–39 sol.	517	607	592.5–621.7	29.2	607.1	±	±
140	Phlox. Tol. Blue, 12–18–40 sol.	530	618	596.4–638.8	42.4	617.6	±	±
122	CWr-13, 6–10–40 sol.	515	620	601.4–641.7	40.3	621.5	+	+
151	Leishm. NAC No. 9034, 5–6–39 sol.	515	622	603.8–637.7	33.9	620.7	++	+
141	Old War pooled, 12–18–40	517	628	602.4–655.0	52.6	628.7	++	+
135	NWr-14, 9–16–40 sol.	516.5	627	606.6–650.4	43.8	628.5	++	±
142	Balch 11–21–38, 12–18–40 sol.	516	624	604.8–662.2	57.4	633.5	++	++
132	McNeal, 5–1–36 sol.	516.5	634	604.3–652.3	48.0	628.3	++	+
134	Leish. Gr. JWM, 12–18–40 sol.	518	633	599.1–662.8	63.7	631.0	±	±
136	NWr-4, 12–18–40	517	635	605.2–660.6	55.4	632.9	+	+
137	NWr-13, 12–18–40 sol.	517	656	617.2–669.8	52.6	643.5	++	++
146	Leishm. BW, 12–20–40 sol.	517	652	630.5–665.9	35.4	648.2	++	+
149	AzIIE Gr. B, 12–20–40 sol.	517	656	636.4–669.0	32.6	652.7	++	++

133	Leish. Gr. P., 12–18–40 sol.	515	656	640.3–668.8	28.5	654.5	++	+ to ++
144	NWr-10, 12–18–40 sol.	517	658.5	640.1–670.2	30.1	655.1	++	+++
145	CWr-12, 12–18–40 sol.	517	660	645.6–671.5	25.9	658.5	++	++
123	NWr-15, 10–18–40 sol.	516	660	641.1–671.5	30.4	656.3	++	++
143	Hastings NAC 8589, 12–18–40 sol.	516.5	660	642.5–671.7	29.2	657.1	++	+++
127	Wright "H-O", 12–16–40 sol.	517	659	648–670	22.0	659.0	++	++
148	AzIIE Gr. A, 12–20–40 sol.	517	661	647.2–671.4	24.2	659.3	+ to ++	+
150	AzIIE Gr. C, 12–20–40 sol.	517	662	648.7–670.9	22.2	659.8	irr. +	+
125	LWr-9, 12–20–39 sol.	516	661	648.0–671.5	23.5	659.7	++	+
138	H-L Co. Recent, 12–18–40 sol.	516	662	648.7–671.8	23.1	660.2	+++	++
147	Leishm. EA, 12–20–40 sol.	517	663	650.0–671.6	21.6	660.8	irr. +	+
161	LWr-10, 1–16–41 sol.	516	664	649.4–671.7	22.3	660.5	++	++

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thru out which D is at or over 90% of its value at λ.
†In this column symbols signify as follows: ±, poor; +, fair; ++, good; +++, excellent.

TABLE 3. SPECTROPHOTOMETRIC DATA AND STAINING PERFORMANCE OF EXPERIMENTAL BATCHES OF WRIGHT'S STAIN

Graph No.	Lot number and procedure	Absorption maxima		Wave-length band of 90% D λ			Staining performance†	
		Eosin	Thiazin	Range λ	Width	Median λ	Blood	Plasmodia
162	WA open beaker, water bath	516	656	636.4–668.6	32.2	652.5	+++	++
163	WB Erlenmeyer, water bath	517	656	639.1–669.9	30.8	654.5	+++	+++
164	WD open beaker, water bath, neutralized	516	654	625.5–667.8	42.3	646.6	+++	+++
165	WE open beaker, water bath	517	650	613.6–665.6	52.0	639.6	+++	++
166	WF Erlenmeyer, water bath	516	654	630.6–668.7	38.1	649.6	+++	++
167	WG Erlenmeyer, boiled, reflux, neutralized	515	617	601.7–632.0	30.3	616.8	—	—
168	WH open beaker, water bath, neutralized	516	657	628.0–669.0	41.0	648.5	+++	+++
169	WI small closed flask, Arnold	517	656	637.7–669.0	31.3	653.3	+++	++
170	WK large open flask, Arnold	516	625	604.6–662.4	57.8	633.5	+++	+

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: D = log 1/T.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thru out which D is at or over 90% of its value at λ.
†In this column symbols signify as follows: +, fair; ++, good; +++, excellent.

The former is in the zone of the absorption maxima of thionin (598–602), azure C (611) and methylene violet (3 samples at 602, 608 and 610 mμ respectively). In our previous studies none of these three dyes gave satisfactory leucocyte or parasite staining, and the performance of the year-old Wright stain solution is in accord.

To test this variability further, 1 g. samples of the same lot of methylene blue (LA-7) were polychromed 1 hour with 100 cc. of 0.5% $NaHCO_3$, two (WA, WE) in open 800 cc. beakers in a bath of boiling water, two (WB, WF) in Erlenmeyer flasks in a bath of boiling water, two (WC, WG) in Erlenmeyer flasks boiling under a reflux condenser, one (W1) in a closed 120 cc. Erlenmeyer and one (WK) in an open 500 cc. Erlenmeyer in the Arnold steam sterilizer, and two (WD, WH) in 800 cc. beakers in a water bath with subsequent acetic acid neutralization. All were cooled, filtered, and precipitated with sufficient eosin Y to give a light blue solution between the precipitate granules (Lot LE-11). The precipitates were filtered out, dried and dissolved 0.1 g. to 80 cc. C. P. methanol and spectrophotometric studies and staining tests were made as before. The results are presented in Table III.

Lots WC and WG developed precipitates which filtered out. In WC only the filtrate was used and no eosinate precipitate was formed. In WG, 0.35 cc. glacial acetic acid was added to 100 cc. distilled water and the precipitate dissolved in it and then combined with the filtrate before making eosinate. It was necessary to add as much as 80 cc. water each to lots WA, WD, WE and WH to compensate for evaporation. Separate spectrophotometrographic examinations were made of the filtrate and the redissolved precipitate of lot WG. The 90% maximum absorption bands ranged from 585.5 to 621.5 for the filtrate and 600.7 to 635.6 for the precipitate, with medians at 603.5 and 618.1 respectively, and ordinary absorption maxima at 603 and 614–622. These were combined to prepare the eosinate solution of WG.

The variability in results is more evident in the width and median point of the 90% of maximum absorption band for the thiazin component than in the maxima themselves. Thus the four open beaker samples with thiazin absorption maxima at 656, 654, 650, and 657 mμ gave band widths of 32.2, 42.3, 52.0 and 41.0 mμ, and medians at 652.5, 646.6, 639.6 and 648.5 mμ. The two Arnold specimens showed the greatest variation, maxima at 656 and 625, bands of 31.3 and 57.8, medians at 653.3 and 633.5 for the closed and open flasks respectively.

TWO CONVENIENT WASHING DEVICES FOR TISSUES AND SLIDES

GEORGE H. MICKEY AND HOWARD TEAS, *Dept. of Zoology*,[1] *Louisiana State University, Baton Rouge, La.*

ABSTRACT.—**Two improved mechanisms are described: (1) For washing fixed tissues, a device which utilizes a downward movement of water with a consequent thoro washing is most convenient, because the washing tubes may be removed or replaced instantly. (2) A pan for washing microscope slides, fitted with a valve stem from an automobile inner tube, produces unusually effective washing and accommodates as many as 80 slides at once.**

1. MECHANISM FOR WASHING TISSUES

Altho useful methods of washing fixed tissues have been reported by Turner[2] and Wetmore[3] the device described here is recommended because of its unusual convenience and effectiveness.[4]

Its convenience is due to the extreme ease and simplicity of operation. Once the device is constructed, one needs merely to place the tissue in the glass washing tube and clip into position. The mechanism is very compact, light, and easily handled; and if made from aluminum, will not discolor a porcelain sink with rust.

The effectiveness of the device is dependent upon its principle, which is similar to that of the method suggested by Galigher[5], who points out two distinct advantages over other methods: (1) "*all* the water passes over the tissue," and (2) "the water moves *downward* so that gravity aids rather than hinders the removal of heavy salts from the material."

Construction of the washing device is not a difficult matter nor is it expensive, altho the current need of aluminum for defense purposes makes that material harder to obtain. The mechanism is very sturdy

[1]Contribution No. 57.

[2]TURNER, J. P. A simple apparatus for washing Protozoa. Science, **74**, 611–17. 1931.

[3]Wetmore, R. H. The use of celloidin in botanical technic. Stain Techn., **7**, 37–62. 1932.

[4]Since this paper was sent to press a somewhat similar apparatus was described by Kersten, H., and Smith, G. F. An apparatus for washing tissue. Stain Techn., **16**, 157–64. 1941.

[5]Galigher, Albert E. The Essentials of Practical Microtechnique. Albert E. Galigher, Inc., Berkeley, Cal. 1934. (See pp. 78, 79).

and should last indefinitely with the possible replacement of a clip after long usage. One needs only an aluminum baking pan 5½ × 10 × 3 in., a piece of ¼ in. aluminum tubing 12 in. long, one piece of angle aluminum 12 in. long, about 48 in. of strip aluminum, and 15 copper rivets with their washers. Small machine bolts might be substituted for the rivets altho, unlike the copper, they would rust.

Details of construction are indicated in figures 1, 2, and 3. Figure 1 is an end view showing the way in which the aluminum tube (t) is held to the arched support (su) by a fitted strip riveted underneath. It also shows the relationship of the angle piece (an) to the clamps (cl) which hold the glass tubes (g). The same feature is shown from top view in figure 3. The angle aluminum is bent over the ends of the pan and riveted in position. By twisting a loop of fine stainless wire around the hilt of the clamp (fig. 3, w) the springiness is adjusted and preserved. Several small holes are punched in the metal tube with a large darning needle after the glass tubes are placed in position so that the holes will be sure to lie immediately over the latter. It is helpful also to place the holes at such an angle that the water is thrown toward the periphery rather than the center of the glass tubes. One end of the aluminum tube is connected to a water spigot by means of rubber tubing (r), while the other end is closed by bending over tightly or by inserting a cork.

The mechanism, as shown in figure 2, will accommodate five glass washing tubes simultaneously, a number which is adequate for the usual laboratory needs or even for small classes in technic. The glass tubes, which are 1 in. in diameter and 2 in. long, may be made of glass tubing or from shell vials with the bottoms removed. They should have both ends spread slightly like the bulb end of a medicine dropper; the upper end to guard against possible slipping thru the clamp, the lower end to hold the silk bolting cloth or cheese cloth which may be held in place by a string or rubber band. The method may be adapted to any size of material merely by changing the mesh of bolting cloth.

The glass washing tubes can be removed or replaced quickly and easily and may be adjusted to any height desired. When they are raised nearly to the aluminum tube, the fine spray of water from the holes in the latter does not strike the tissue with any force and yet gravity causes a constant and complete change of water around the tissues.

2. Washing Pan for Slides

Extreme simplicity, convenience and effectiveness characterize the pan designed for washing microscope slides.

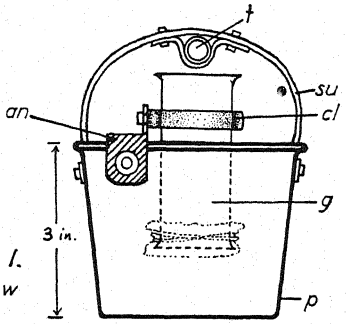


FIG. 1. End View

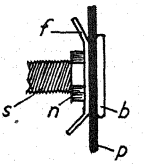


FIG. 4.

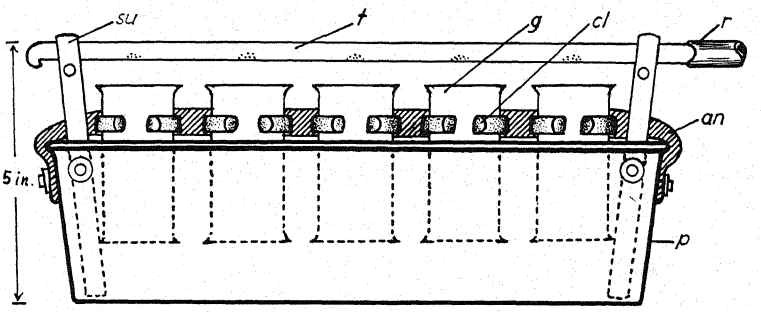


FIG. 2. Side View

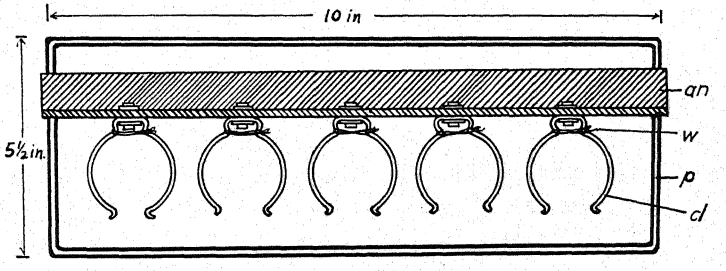


FIG. 3. Top View

FIGURES 1–3. Device for washing fixed tissues.

an. Angle piece
cl. Clamp
g. Glass tube
p. Pan
r. Rubber tubing
su. Arched support
t. Tube
w. Wire

FIGURE 4. Details of construction of pan for washing slides.

b. Base
f. Flanged washer
n. Nut
p. Wall of pan
s. Valve stem

An aluminum baking pan ($5\frac{1}{2} \times 10 \times 3$ in.) is fitted at one end near the bottom with a valve stem from a discarded automobile inner tube. The old style stem with a nut that screws on the outside is necessary. The base (fig. 4, b) and flanged washer (f) have a groove and ridge arrangement which make a water tight joint with the aluminum pan (p) when the nut (n) is screwed down tightly. The valve stem proper (s) should be sawed off to about $\frac{3}{4}$ in., leaving just enough for a good junction with rubber tubing to the water spigot.

A pan of this size will accommodate two large monel metal slide staining racks[6] holding 25 slides each or eight of the smaller racks which hold ten slides each. If one uses, for stains and alcohols, tall Stender dishes into which the small racks fit, he can dispense with practically all handling of individual slides. Placing slides in the rack (after affixing the sections), examining one or two in order to control differentiation, and finally mounting under cover glasses constitute the only individual handling necessary; thus the staining time is cut down considerably.

The washing is unusually effective since water enters the pan from the bottom, passes directly around and between the slides and finally flows over the rim of the pan.

NOTE.—Numerous advantages of aluminum over other construction materials are obvious, but since aluminum is now hard to get, some substitutes might be suggested. Tin baking pans of the same size are available at any five-and-ten-cent store but they would have to be covered with varnish or enamel or asphalt paint to prevent their rusting. Aluminum enamel is the best coating but it is as scarce as the metal itself. Brass or copper tubing could be used instead of aluminum tubing but would necessitate the use of a drill in place of a darning needle for making the holes. Instead of the strip aluminum used for the supports and clamps, one could substitute either copper, galvanized iron, or tin-plate, altho the latter would have to be protected from rusting. Soldered joints could replace the rivets with any of these substitute materials.

[6]Central Scientific Co. catalogue numbers 48060 and 48070, monel metal staining racks for holding microscope slides for staining by immersion.

TECHNIC FOR PHOTOGRAPHING EARLY CLEAVAGE STAGES OF THE HEN'S EGG

M. W. Olsen, *Dept. of Poultry Husbandry, University of Maryland, College Park, Md.*

Altho photographs have been secured of early cleavage stages in the pigeon's egg, no photographs of corresponding stages of development of the hen's egg have been published. Satisfactory photographs of early cleavage stages of the hen's egg have been difficult to obtain in the past because of the indistinctness of the furrows and the lack of contrast between them and the remainder of the blastodisc.

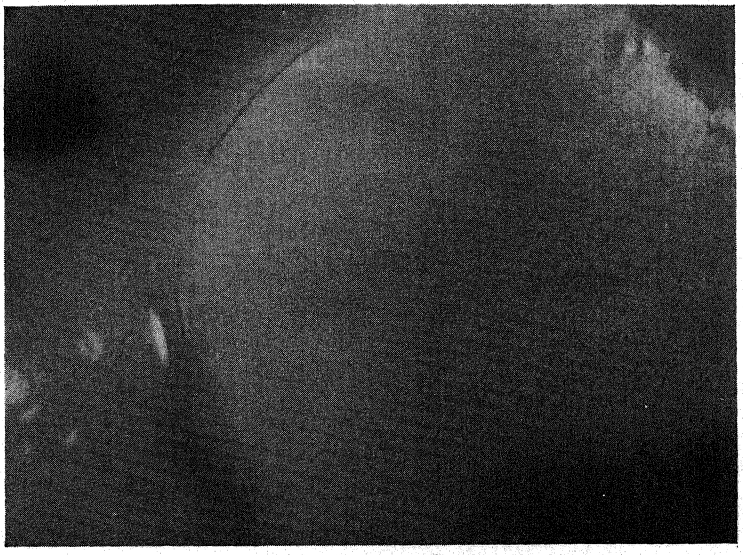


Fig. 1. Photograph of an unstained blastodisc of a hen's egg showing an 8-cell cleavage stage.

The indistinctness of the cleavage furrows in the hen's egg as compared with those of the pigeon's egg was recognized by Patterson (1910) who, after having studied both types of eggs, states (p. 109): "The lack of a furrow is the cause of the indistinctness of the early cells. In this respect, the early cleavages of the hen's egg differ greatly from those of the pigeon's egg, for in the latter their clearness is such as to permit photographing the living cells, while in the former photographs are impossible, except in a few cases".

A number of the photographs of early cleavage in pigeon eggs published by Blount (1909) and reproduced by Lillie (1919) were obtained of the living egg, one of which was taken thru a window in the shell, while the others were secured after the intact yolk was immersed in normal saline solution. Other photographs published by Blount were taken after the blastodiscs were killed and while the yolks were immersed in the killing fluid. Still other photographs were taken from whole-mount preparations, but the author makes no mention of either removing the vitelline membrane or staining the blastodisc before photographing.

During a recent study of the early development of the embryo of the domestic fowl, a technic was devised which makes it possible

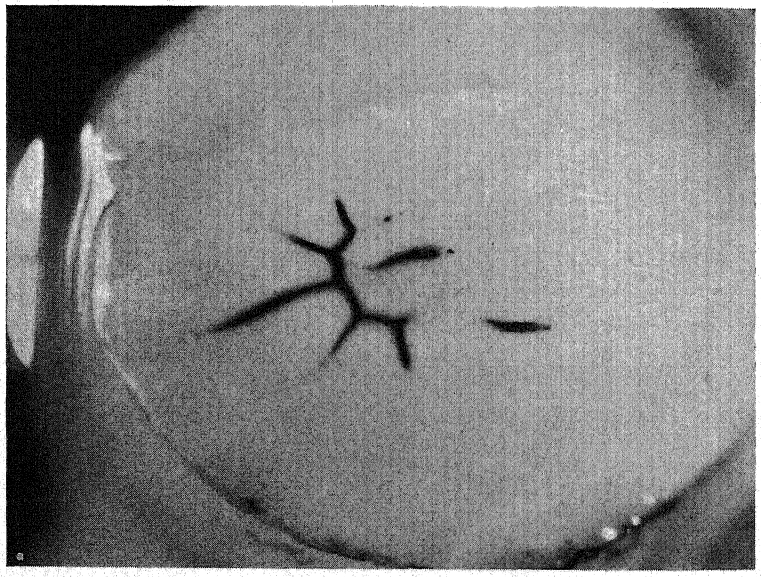


FIG. 2. Photograph of the same blastodisc as shown in Fig. 1, stained with gentian violet before photographing.

to study and to photograph whole-mount preparations of early cleavage stages of the chick embryo. The following procedure was used in preparing the tissue for photographing.

The entire yolk of the egg containing the blastodisc to be photographed is placed in Bouin's fixative and allowed to remain in this solution from 24 to 48 hours. It is important that all the albumen adhering to the vitelline membrane about the blastodisc be removed before fixing the yolk; otherwise difficulty will be experienced in locating the blastodisc. After fixing for 24 to 48 hours, the yolk is

removed from the fixative and placed in 60% alcohol. The vitelline membrane is then removed by means of small forceps. If care is taken in removing the vitelline membrane, the blastodisc is exposed and left undamaged on the yolk. The blastodisc is now removed by cutting about the periphery and beneath the disc with a small pair of scissors. The blastodisc, after being removed from the yolk, is placed in the cell of a depression slide. Two or three drops of 5% gentian violet[1] in 95% alcohol are then applied to the upper surface of the blastodisc. The tissue is then destained by the application of several changes of 60% alcohol. If the destaining process is done carefully, a point can be reached where the stain will be completely removed from the upper surface of the cells, leaving only a small amount of stain in the cleavage furrows. This creates a marked contrast between the cells and the cleavage furrows so that they can be easily photographed. If the destaining is carried too far, the tissue can be restained and the destaining operation repeated.

Photographs of the same blastodisc of a hen's egg before and after preparation by the method just described are shown in Figs. 1 and 2. It will be observed that where the special technic was used (Fig. 2) the cleavage furrows stand out sharply and in marked contrast to those found in the unstained balstodisc and shown in Fig. 1.

After photographing, the blastodiscs can be preserved as semi-permanent preparations by replacing the 60% alcohol used in the destaining process with Canada balsam.

REFERENCES

BLOUNT, M. 1909. The early development of the pigeon's egg with especial reference to polyspermy and the origin of the periblast nuclei. Morph., 20, 1–64.

LILLIE, F. R. 1919. The development of the chick. *2nd Ed.* Henry Holt and Co., New York.

PATTERSON, J. T. 1910. Studies on the early development of the hen's egg. I. History of the early cleavage of the accessory cleavage. Morph., 21, 101–34.

[1]Coleman and Bell's "gentian violet, improved" (a methyl violet 2B) was employed in this work.

A SIMPLE METHOD OF TRANSFERRING TISSUES

M. W. Olsen, *Poultry Department, Maryland Agricultural Experiment Station, College Park, Md.*

It is a common practice among histologists while preparing tissues for sectioning to transfer the tissues from one reagent to another by

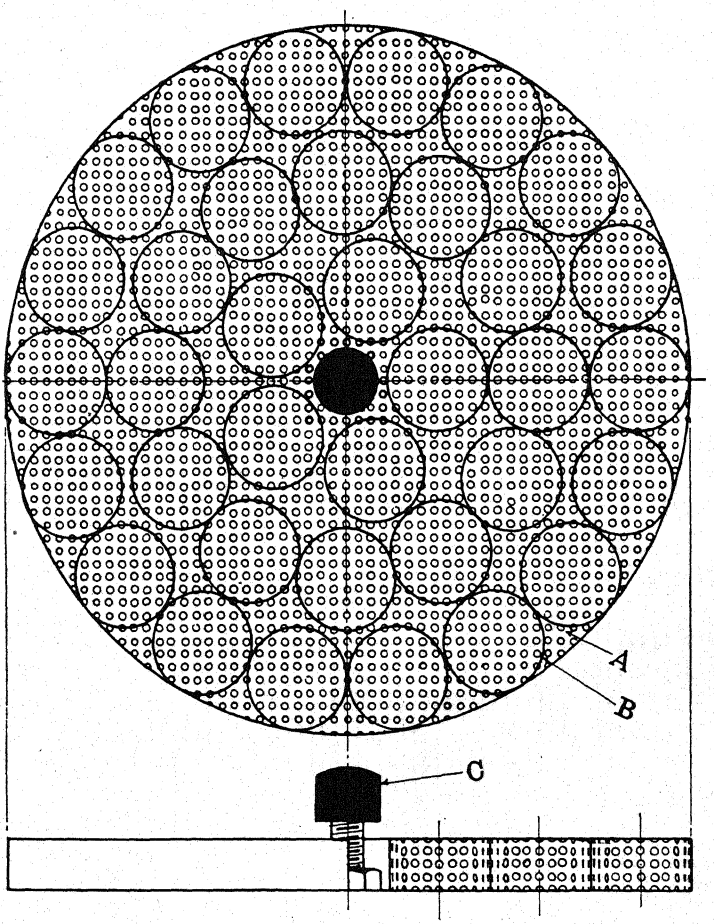


Fig. 1. Carrier used for transferring tissues. It is composed essentially of a base plate (a), individual tissue chambers (b), and a handle (c).

means of small forceps or a spatula. This method can be used safely when the type of tissue being studied is of such a nature that it will

not be injured by these operations. When, however, the tissue is fragile, extreme care must be exercised, if the above method is employed, to prevent damage. In a recent study of maturation and and fertilization of the hen's egg, difficulty was experienced due to breakage of blastodiscs while transferring them from one reagent to another. In order to reduce the chances of damage and to facilitate and simplify the operation of transferring these fragile tissues, the tissue carrier described in the following paragraph was designed.

The tissue carrier is shown in the accompanying illustration. It consists essentially of a perforated metal plate, A, 3½ inches in diameter, to which are soldered 35 perforated circular metal bands. Each band is ½ inch in diameter and ¼ inch in depth and forms with the base plate the individual tissue chamber, B. The base plate and the bands are made of number 30-gauge monel metal having 225 or more perforations per square inch. Other perforated metals may be substituted for the monel metal but in such a case it is advisable, after the carrier has been constructed, to have it plated with some non-corrosive metal such as silver, chromium, or cadmium. A small, wooden knob, C, attached to the head of a ⅛-inch brass bolt serves as a handle. The bolt is screwed into a nut which is soldered to the upper surface and at the center of base plate A. The depth of the tissue carrier, including the handle, is $\frac{7}{16}$ inch. The entire carrier will fit into a 4-inch Petri dish, which can then be covered so as to prevent evaporation of the reagent being used.

The tissues to be dehydrated, cleared, and infiltrated are placed in the small perforated chambers. Once the tissues are placed in their respective compartments they need not be handled individually again until the time of embedding. In order to identify individual tissues, a small slip of paper labeled with laboratory ink is placed in each chamber. To transfer the tissues the carrier is lifted from one solution and placed in a second Petri dish containing the next reagent. This method greatly reduces the chances of damaging the tissues and makes it possible to transfer a large number of tissues quickly and easily with one operation from one reagent to another.

THE PLASTIC ETHYL METHACRYLATE IN ROUTINE LABORATORY TECHNIC

MARY S. CARBONE AND DONALD J. ZINN, *Osborn Zoological Laboratory, Yale University*

ABSTRACT.—The use of ethyl methacrylate as a permanent, transparent, colorless medium for mounting *in toto* invertebrate and vertebrate embryos and small adults is demonstrated.

The liquid monomer is partially polymerized by heating over a hot-plate, the reaction being aided by the use of benzoyl peroxide as a catalyst. The object to be mounted is fixed, stained, dehydrated, and cleared in the usual manner, and is then infiltrated with the partially polymerized ethyl methacrylate. It is oriented on a hardened base in a glass or porcelain dish, and the mold is then allowed to harden (polymerize) completely at a temperature of 38–40° C. under a 250-watt infra-red, ultra-violet bulb. After the block is removed from the dish it may be polished by the ordinary metallurgical methods. More than 40 species of animals have been satisfactorily treated in this manner.

The great need for a satisfactory method of mounting various small invertebrates and vertebrate adults and embryos, as well as transverse or longitudinal sections too large or too thick for mounting on ordinary slides, has long been felt. Of the few technics that have been mentioned in the literature, there appears little doubt that the best is to employ ethyl methacrylate, a resinous plastic soluble in absolute ethyl alcohol, ether, dioxan, oil of wintergreen, xylene, and most soluble in chloroform. The use of a similar material, methyl methacrylate, has been suggested by Hibben and by Cole[1], while Puckett[2] has worked with ethyl methacrylate, but neither of these authors has demonstrated the apparent general application of these plastics to all small, cleared metazoa. There are, in addition, certain refinements in technic developed by the authors which have simplified the process and made the creation of satisfactory clear blocks more certain.

[1]Cole, Elbert C. 1938. Methyl methacrylate as a laboratory tool. Science, 87, 396–8.

Hibben, J. H. 1937. The preservation of biological specimens by means of transparent plastics. Sci., 86, 247–8.—A recent personal communication from this author indicates that he has now worked out a more complete method for mounting specimens in methyl, ethyl, and butyl methacrylates.

[2]Puckett, W. O. 1940. Ethyl methacrylate as a mounting medium for embryological specimens. Science, 91, 625–6.

The advantages of a permanent, transparent, colorless, easily prepared mounting medium are considerable (a) from the point of view of the teacher and the museum preparator, (b) for the personal use of the student in the laboratory and (c) for the permanent museum demonstration. The problems of the storage of specimens, cost of slides, jars and other containers disappear, and the potential breakage and color-changes of the preserving fluids are virtually negligible. Whelan[3], in his comprehensive review of the use of various plastics in entomology, states with regard to the methacrylate resins—"The solid form possesses extraordinary colorless transparency, stability against aging, and chemical resistance to many reagents". The advantages of ethyl methacrylate (Plexiglas), manufactured by the Röhm and Haas Company, Philadelphia, Pa., ($6.00 per gallon) are demonstrated in the greater ease with which it may be handled in the preparatory and in the hardening processes.

The liquid monomeric ethyl methacrylate is now shipped in a pure state; i.e., it does not contain an inhibitor (Puckett, op. cit.). Shipment in dry ice and keeping the material in a refrigerator at a very low temperature prevent spontaneous polymerization. Puckett, as well as Sando[4], worked with material which was kept in the monomeric state by means of an inhibitor, hydroquinone, which either had to be washed out with KOH, or had to be separated by distillation. Since it was found possible to keep the liquid resin as a monomer by means of refrigeration, the use of an inhibitor was eliminated by the manufacturer. And because light and heat aid in spontaneously polymerizing the monomeric ethyl methacrylate, only the amount of material which is to be used should be prepared.

Our procedure is as follows:

To 500 cc. of the monomer in a liter Florence flask are added 50 cc. of a catalytic solution, made by dissolving 5 g. of benzoyl peroxide in 100 cc. of the monomer. The flask is stoppered with a cork thru which a thermometer has been inserted, as well as a glass L-tube drawn to a fine point to allow for dissipation of heat generated in the polymerization reaction. An electric hot-plate is used rather than a water-bath or any device which necessitates the use of a flame, as the material is inflammable, and its polymerization may easily get out of control. To prevent too rapid a polymerization, the temperature of the hot-plate is adjusted so that the methacrylate solution will be between 83° and 85°C. While the reaction is taking

[3]Whelan, Don B. 1941. The role of plastics in the field of entomology. Kansas Entom. Soc., 14, 73–89.

[4]Sando, Charles. Embedding Specimens in Plexiglas. (Dist. by Rohm and Haas Co., Phila., Pa.)

place, the flask is shaken every few minutes in order that excess heat be liberated, and thus boiling over of the solution is prevented. At 85°C. the methacrylate becomes as viscous as syrup. When this point is reached, the flask is uncorked and plunged into ice-water. After having cooled, the flask is well corked, and stored in the refrigerator until the solution is needed.

Both vertebrates and invertebrates have been mounted in this medium: chick, pig and rat embryos, turtles, frogs, fish, tunicates, amphioxus, bryozoa, hydroids, flatworms, roundworms, brittle-stars, molluscs, spiders, 41 species in all. These animals are fixed in alcohol, formalin, or other fixatives; and then stained, dehydrated, and cleared as for ordinary mounts.

Dishes made of glass, ceramics, tin, and brass may be used as molds. The glass and ceramic containers prove best from the point of view of: (1) the ease with which the polymerized methacrylate was removed from the mold, and (2) the finish of the block. The molds, half-filled with the partially polymerized solution, are tightly covered, with cork or glass, and placed in an oven at 50°C. for 24–48 hours. This final heating causes the ethyl methacrylate to be polymerized completely, and forms a hard base on which to mount the object. During this process, the solution evaporates about 50%.

The animal to be embedded is placed in a dish of the partially polymerized material, which in turn is set in a desiccator for 12-24 hours to allow for evaporation of the clearing agent and to permit infiltration. Just before placing the animal in the mold, the dish is filled with the partially polymerized material and then allowed to stand so that the fresh solution will slightly soften the base. This step serves to facilitate orientation, since the object may move during the final heating if it is not held fast in the softened surface of the base. A small India ink label may now be included.

The mold should be treated in either of two ways. The better method for the final polymerization reaction involves the use of a strong infra-red, mild ultra-violet bulb.[5] The bulb is suspended about 10 inches over the dish, which is kept slightly uncovered. The heat generated by the lamp reaches 38°–40°C., and the length of time required to harden a block depends upon its size. For example, a block 6.5 cm. in diameter and 1.5 cm. thick will harden in 20 hours. The other technic requires that the methacrylate be finally polymerized in an oven at 50°C. The authors prefer the former method because the block can be watched more readily during the polymerization.

[5]General Electric Mazda CX, G-30; 250 w., 120 v.; Strong infra-red, mild ultra-violet; 4750 Lumens; $1.25.

After the block has hardened, and while the dish is still warm, the edge of the mold is freed from the walls of the dish with a knife, and the dish is plunged into icewater or put into the refrigerator until the entire block is loosened.

The molds are easily shaped and polished. The final shape of the block may be formed with a hacksaw. A flat machinist's file is used to remove deep surface cuts, and a finer file (magneto file) is employed to smooth this surface. Final polishing is accomplished on a revolving wheel by using a simplified metallurgical polisher with fine polishing alumina such as that manufactured by the Fisher Co.[6]

If air is trapped in the object or in the block during the final polymerization, or if a thick froth forms which will harden when not noticed in time, the specimen may be rescued by dissolving the polymer in chloroform, and the embedding procedure repeated.

Because of the nature of the final polymerization of the plastic, there are certain conditions under which particular caution must be observed. This concerns especially the embedding of soft-bodied invertebrates such as those of the genera, *Lumbricus*, *Dolichoglossus* and *Placobdella*, in which the epidermis is sometimes pulled apart. The authors were not able to correct this. In addition, slight shrinkages were noted in the exoskeletons of several species of the Arachnida. Of the stains successfully used during this experiment, borax carmine and Feulgen tended to fade slightly. Colored clearing agents such as beechwood creosote were found unsatisfactory, as they imparted a yellow cast to the finished block.

[6]Fisher Polishing Alumina, Grade No. 3, Fisher Scientific Co., Pittsburgh, Pa.

A SCHEDULE FOR CHROMOSOME COUNTS IN SOME PLANTS WITH SMALL CHROMOSOMES

B. L. HANCOCK,[1] *Department of Botany and St. John Baptist College, Oxford, England*

ABSTRACT.—A schedule is given which produced excellent results for somatic chromosome counts on some British species of *Galium*, These species present great difficulties owing to the small size of the chromosomes (ca. 1.5μ long), the large somatic numbers (up to 96) and the slenderness of the root tips. Special features of the schedule, which is the result of much experiment with various technics, are: fixation with Belling's Navashin type fixative, which was quite the best tried; the modification of Randolph's card mount method to overcome the difficulty of the small diameter of the root tips by mounting together a number from the same plant, so that they can be embedded and sectioned almost as easily as much larger root tips; staining with dilute (0.1%) crystal violet, the most critical stain used, after mordanting with 1% aqueous chromic acid to intensify the stain in the small chromosomes; and the addition of an extra stage of differentiation in absolute alcohol diluted with xylol to remove strands of stain, which are often left in the cytoplasm between the chromosomes, since clove oil, usually the last differentiating fluid used, differs from alcohol in removing crystal violet more rapidly from the chromosomes than from the cytoplasm. It is suggested that this schedule will be valuable in chromosome counts of other plants where similar difficulties arise.

The British species of *Galium* have very small chromosomes (ca. 1.5μ long) whose number may be as high as 96, and they therefore present great difficulties for the cytologist. There is need for the utmost care in fixation owing to the high magnification that must be employed, and also to the large number of chromosomes commonly found in these species; and there are also difficulties in bringing about an adequate staining of the chromosomes. A further source of trouble is that the root tips, which were the only suitable parts of the plants for chromosome counts at the time the work was done, are of very small diameter, being little thicker than hairs when growing in soil.

Among numerous fixatives tried, Belling's Navashin type fixative

[1]Present address: Imp. College of Trop. Agriculture, St. Augustine, Trinidad, B.W.I.

(formula in La Cour, 1937, and used with the larger proportion of formaldehyde) was the only one to give excellent results. The chromosomes stained well, were usually well spaced, and were slightly swollen and shortened, a fact which facilitated counting. After Randolph's CRAF, the chromosomes were longer, often with a tendency to clump together, and were less intensely stained. Medium Flemming's fluid resulted in a still more inferior fixation, altho the chromosomes were swollen and intensely stained, rather as after Belling's fixative. La Cour's 2BE and 2BD gave variable staining, some metaphase plates being unstained, while others, even in the same preparation, were quite well stained. Apart from this serious drawback, 2BE fixation was quite good. Modifications of some of these fixatives were tried, but they either gave similar or inferior results.

FIG. 1. *Galium palustre* (diploid, 2n=24) treated according to accompanying schedule. × ca. 4000.

The difficulty of the small diameter of the root tips was eased by growing the plants in tap water, which was changed daily to improve aeration. Then considerably stouter and straighter adventitious roots were produced in abundance, especially by the marsh species. Even so, the root tips were so slender that something like 10 or 15 roots had to be sectioned, especially in the case of plants with 96 chromosomes, to be relatively certain of obtaining an excellent metaphase plate. To achieve this without undue labor, all the root tips had to be orientated parallel to one another in the melted wax, but their small size made this rather tricky operation particularly difficult. Accordingly Randolph's card mount method (1940) was modified to obviate these difficulties. His method was followed fairly closely, using for gum Stephen's "strongest mucilage". The root tips were hardened in 50% alcohol and then mounted; a number from the same plant were stuck parallel and close together so that they supported one another by surface tension. They were handled by means of small forceps, and were not dried before mounting, because the small amount of alcohol carried with them was useful for its surface tension effect, and essential to keep the delicate tissues from drying up. Once mounted, it is a simple matter to transfer the whole card from one fluid to another without danger of loss, such as is present when the minute root tips are not attached to one another. Incidentally this method allows economy in dehydrating fluids, since a number of mounts from different plants can be treated

together. The times of dehydration and infiltration should be longer rather than shorter owing to the mass of paper which should also be infiltered.

The staining schedule was modified from Randolph's procedure quoted by Newcomer (1938). Dilute (0.1%) crystal violet was found to give best results, and his mordanting with chromic acid before staining was employed in order to intensify the stain in the small chromosomes. A rather longer time (5 minutes) was given in the alcoholic iodine solution. Iodine treatment of less than 3½ minutes

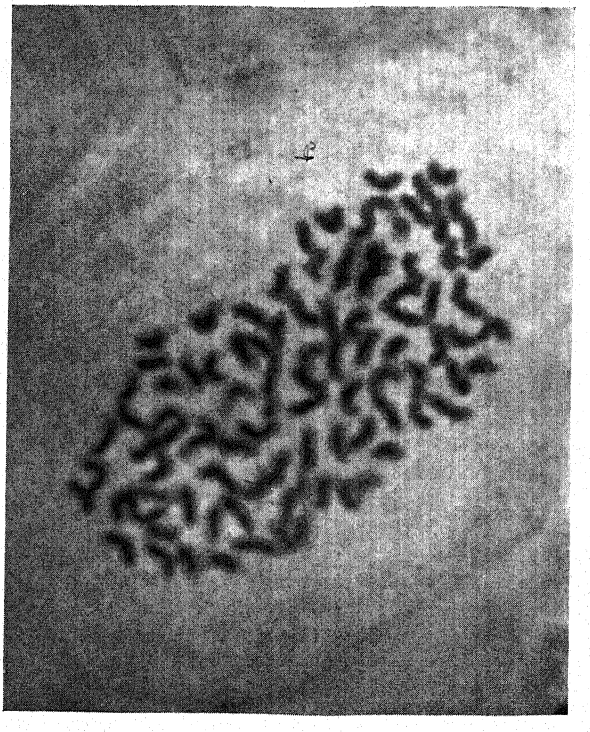


FIG. 2. *Galium palustre* (octoploid, 2n=96) treated according to accompanying schedule. × ca. 4000.

usually resulted in stain being retained in the cytoplasm, while the chromosomes were, in general, stained lightly after more than 7 minutes in the iodine solution. There was, however, considerable variation in this behavior, suggesting that the optimum time varies with the species and the treatment. An important addition to the schedule was about a minute spent in the mixture of xylol and alcohol; this resulted in the clearing away of strands of stain, which otherwise

were usually left in the cytoplasm between adjacent chromosomes, making interpretation more difficult. These strands of stain appear to be a result of clove oil, since it removes the stain more rapidly from the chromosomes, and it is the last differentiating fluid used. The extra stage in alcohol diluted with xylol removes them without reducing the intensity of the stain in the chromosomes, and therefore increases the contrast.

Other stains tried were Heidenhain's iron-alum hematoxylin and Feulgen. Neither of these was so critical, altho the latter has been used in many different ways, including Warmke's section-smear technic (1941). Some difficulty was experienced in staining the chromosomes with sufficient intensity, but partial success was achieved following his schedule with 5 hours in the stain and 3 minutes in each of the sulphite solutions, while the contrast was improved by 10 minutes in running tap water after staining, as recommended by Warmke. The main drawback to this technic was that pressure stretched out the constrictions, greatly separating small parts of the chromosomes, and making interpretation very difficult. In addition, altho the more evenly spaced chromosomes were easily separated by pressure, groups of overlapping chromosomes, whose interpretation was in doubt, were much more difficult and sometimes impossible to separate.

The following is the complete schedule:

(1) Fix the root tips in Belling's Navashin type fixative. Evacuate for a few minutes and leave	overnight
(2) Harden in 50% alcohol	3 hours
(3) Mount root tips on card	
(4) Dehydrate and harden gum in 70% alcohol	overnight
(5) Continue dehydration, infiltration and embedding in paraffin wax by the chloroform method (La Cour, 1937)	a total of about 10 days
(6) Cut sections (transversely for chromosome counts) about 10μ in thickness	
(7) Dissolve out the wax in xylol, and take the slides down from ethyl alcohol to water in the usual way	
(8) Mordant in 1% aqueous chromic acid	20 minutes
(9) Rinse in tap water and then in two or three changes of distilled water	about 10 minutes
(10) Stain in 0.1% (or 0.5%) aqueous crystal violet	2½ hours
(11) Rinse in tap water	
(12) 1% iodine and 1% potassium iodide in 80% alcohol	5 minutes
(13) 95% alcohol	2–3 seconds

(14) Three changes of absolute alcohol	a total of 30–45 seconds
(15) Clove oil	10–15 seconds
(16) Xylol	2 minutes
(17) Xylol, 2 vol., to 1 vol. absolute alcohol	45–100 seconds
(18) Three changes of xylol	a total of about 2 hours

(19) Mount in Canada balsam.

This schedule is relatively short, as long periods of dehydration are eliminated; the longest part is the infiltration, which should not be much less than 8 days, or the card will not be adequately infiltered. Uniformly good results were obtained on the species investigated with the slightly swollen and intensely stained chromosomes standing out, sharp in outline, from the colorless cytoplasm, even under the magnification of 2,700 diameters which was needed for counting. Little stain was retained by the nucleoli or resting nuclei, so that the chromosomes were easily found and were rarely obscured by overlying cell structures.

It is suggested that this schedule may prove to be of value for other species with small chromosomes where similar difficulties arise, and the modification of the card mount method should be valuable for all plants with minute root tips.

REFERENCES

La Cour, L. 1937. Improvements in plant cytological technique. Bot. Rev., **3**, 241–58.

Newcomer, E. H. 1938. A procedure for growing, staining and making permanent slides of pollen tubes. Stain Techn., **13**, 89–91.

Randolph, L. F. 1940. Card mounts for handling root tips in the paraffin method Stain Techn., **15**, 45–8.

Warmke, H. E. 1941. A section-smear method for plant cytology. Stain Techn., **16**, 9–12.

AN INEXPENSIVE APPARATUS FOR CUTTING TISSUE SECTIONS ON THE SLIDING MICROTOME BY THE "DRY ICE" METHOD

M. V. ANDERS, *Brown University, Providence, R. I.*

The use of "dry ice" in the preparation of frozen sections in histological technic has been increasing in popularity in recent years. Current literature largely deals with apparatus adapted for cutting large blocks of tissue, such as transverse or longitudinal sections of whole brains or other large organs, and usually must be constructed

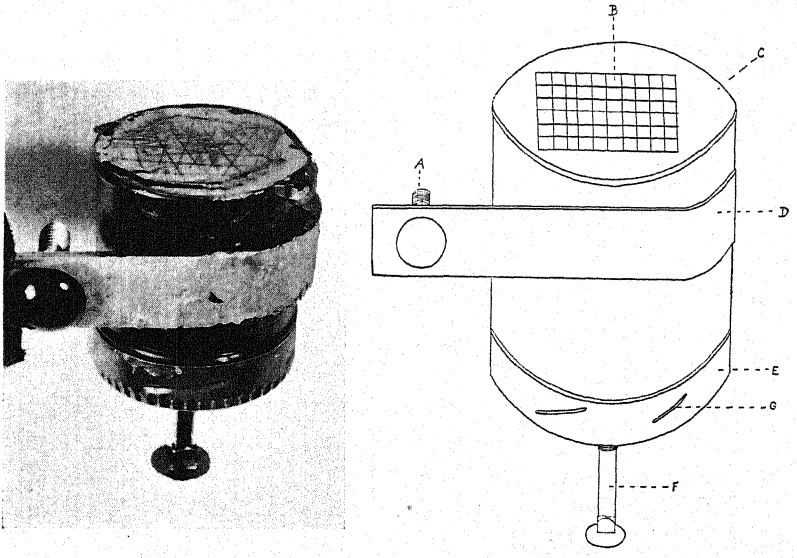


FIG. 1. Photograph and sketch of carrying unit for the "dry-ice", showing the details of construction.

(A) Mounting screw for attaching freezing unit to the microtome micrometer-screw rack.

(B) Piece of grooved tin acting as a freezing surface for the tissue to be cut.

(C) Bottom of can filled to the edges with solder.

(D) Angle iron soldered to side of can for carrier attachment.

(E) Can lid.

(F) Stove bolt acting as plunger to force "dry-ice" up against the bottom of the can for the freezing operation.

(G) Slots and grooves for keeping can lid tightly in place.

as a unit or in part by some particular manufacturer. The apparatus used by the author resembles that used by Dr. J. W. Lind-

say, M.D.[1], but is less expensive and more easily constructed. The necessary materials may be found around the laboratory or may be purchased locally for not more than the total cost of twenty cents.

The container for the "dry ice" is a small tin can, the size of which is governed by the type of work to be done. The one in use at present (Fig. 1) was the container for small lock washers purchased at the local 'dime' store. It is 1¾ inches in diameter by 2 inches high, with a screw-on top. The bottom is strengthened and adapted to act as a freezing surface by coating the outside with a heavy layer of solder, in which is fused a small piece of tin properly grooved to receive the block of tissue. Both the piece of tin and the surrounding solder are made to form a flush surface with the edge of the can. The cap of the can is pierced thru the center by a hole just large enough to carry a 1⅛ inch stove bolt. The nut of this bolt is soldered to the

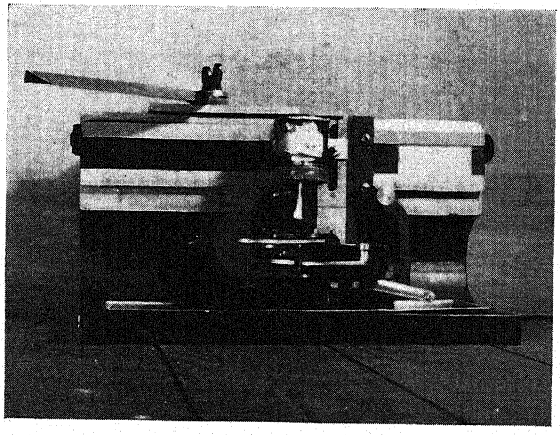


FIG. 2. Freezing unit mounted on a Bausch and Lomb sliding microtome ready for use.

inner surface of the cap to match the hole, permitting the bolt to be screwed thru the cap and adjusted from the outside of the can. A piece of tin or cork may be attached to the end of the adjustable bolt, thus providing a means of continually forcing the "dry ice" up against the surface on which the tissue is to be frozen.

To attach this container to the microtome, a two-inch angle iron may be bent so as to fit snugly around the circumference of the can and soldered in place close to the cap-end. This strap of iron is allowed to project tangentially 1¼ inches from the side of the box,

[1]Lindsay, J. W. 1931. Frozen sections prepared by use of "dry ice". J. Amer. Med. Assoc., 96, 773.

the side from which it projects being governed by the type of microtome for which it is made. It should be made to hold the base of the can upward, or facing the entering edge of the microtome knife. A small stove bolt, selected with the head the correct size to fit the slide-groove of the object-holder of the microtome on which the apparatus will be used, is then put thru the hole which is already in the free end of the projecting strap of iron, and the apparatus is ready for use.

The celloidin-block carrier of the sliding microtome is removed and the "dry ice" apparatus inserted in its place (Fig. 2). The box is filled with small pieces of "dry ice", the stove bolt tightened till the "ice" is in contact with the freezing surface, and the tissue is put in place.

Good thin sections of the spinal cord of small laboratory animals have been obtained consistently with the above apparatus. Both gelatin-embedded tissues and non-embedded tissues have been sectioned equally well. Between one hundred and two hundred sections have been cut with one pound of "dry ice", at the initial cost of but six cents. This is decidedly more economical than the use of liquid carbon dioxide. Good results have been obtained without the use of any insulation, but by lining the box with a thin layer of felt or paper toweling, the apparatus becomes even more economical. If desired, freezing may be hastened by placing small pieces of "dry ice" around the tissue-block for the first minute or two.

An additional advantage of this method is the small amount of space required with the microtomes which are usually available. The sliding microtome has the advantage that the angle of entrance of the blade to the block of tissue may be varied to obtain good sections from tissues of different consistencies. In the case of soft tissues, thinner sections may be cut by the use of a small basket, constructed of a piece of thin wood with a wire clip on either end, which is clamped over the back edge of the microtome knife to carry a piece of "dry ice" to cool the blade.

NOTES ON TECHNIC

Simultaneous Staining with Sudan-Hematoxylin

Mixtures of 70% alcoholic Sudan III with Delafield's or Ehrlich's hematoxylin have been found to stain fat, nucleus and cell structures simultaneously. They keep well and maintain their staining qualities for 6 months or more provided they are prepared with freshly made Sudan. The precipitate appearing after mixing of the two liquids must not be removed.

The method gives good results and has the advantage of being simple and quick. The time can be reduced still further by using ammonia-water (approximately 1 drop of ammonia to each 5 cc. of tap water) for differentiation, a matter of importance for clinical work in rush diagnoses. The risk of losing mounted sections, often encountered with successive staining, is reduced to a minimum.

The sections are brought into the mixture from distilled water or weak alcohol. After staining, they are transferred to water for differentiation. No weak alcohol is required after staining if this is done in a well closed vessel.

The proportions of the mixture depend on the time required for staining the fat. Where 15-minutes' staining is needed, 5 parts of Sudan III with 1 part of hematoxylin have been found satisfactory. Many fats stain within 5 minutes, which enables a considerable shortening of the whole procedure. For such cases a mixture of 3 parts Sudan III with 1 part hematoxylin is required. The procedure is as follows:—

1. Stain in Sudan-hematoxylin..... 5 minutes.
2. Differentiate in ammonia-water..... 15–20 seconds.
3. Tap water..... 15–20 seconds.
4. Enclose in glycerin or glycerin-jelly.—Emil Liebmann, *Dazian Foundation Fellow, Division of Microscopic Anatomy, Tulane University, New Orleans, La.*

An Improved Acid Hemalum Formula

Mayer's acid-hemalum has the advantage over some other alum hematoxylin formulae in its ease and speed of preparation and selectivity of nuclear staining; but solutions are rather promptly exhausted and do not keep particularly well. Increasing the concentration of hematoxylin and adding glycerol to the formula appear

to have remedied these defects. The old and new formulae are as follows:

	old	new
Hematoxylin	1.0 g.	5 g.
$NaIO_3$	0.2 g.	1 g.
$AlNH_4(SO_4)_2+12\ H_2O$	50.0 g.	50 g.
Distilled water	1000.0 ml.	700 ml.
Glycerol	—	300 ml.
Glacial acetic acid	20.0 ml.	20 ml.

The alum is first dissolved in the water, then the hematoxylin, then the iodate is added, and, when the color change has occurred, then the glycerol and the glacial acetic acid. No ripening is necessary.

Very satisfactory staining may be attained in as little as two minutes on ordinary formaldehyde-fixed human surgical material, but a 5-minute interval is usually employed. Bluing for 2–10 minutes in tap water and counterstaining one minute in 0.2% aqueous eosin Y are followed by dehydration in alcohol as usual.

Hematoxylin solutions several months old are apparently as good as fresh. It has not yet been determined just how long it will keep.

This formula and the Weigert acid-iron-chloride formula were recently employed in the testing of 14 different lots of hematoxylin in comparison with the Delafield formula and with Mallory's phosphotungstic acid hematoxylin. All four formulae gave satisfactory results with the same 12 lots and unsatisfactory with the other 2 lots. Delafield formulae were used after 6 and 8 weeks' ripening. Phosphotungstic formulae required 20 weeks for fully satisfactory ripening.

The hematoxylin samples used were the American certified lots FH-10, FH-11, FH-13, FH-14, FH-15, FH-16, FH-17 and FH-18, an uncertified lot from Harmer about 1918–1920, the German lots E&A No. A20–385, E&A No. B115 and Grübler No. 11.38, all of which were satisfactory and Grübler 04.9 and Am. Dyewood No. 251, 1917, which were unsatisfactory.—R. D. LILLIE, *Senior Surgeon, USPHS., National Institute of Health, Bethesda, Md.*

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

KOGA, ARATA, and AMANO, S. **Ultraviolettmikroskopie in der Pathologie. III.** *Trans. Soc. Path. Jap.*, **30**, 115–20. 1940.

This review is based on the use of a microscope with a quartz lens system adapted for the 2750Å cadmium or the 2800Å magnesium line. Ultraviolet microscopy is considered especially useful in bringing out differences based on the aromatic amino acid content of proteins, also porphyrin and nucleic acid groups.—*S. H. Hutner.*

MICROTECHNIC IN GENERAL

KIRKPATRICK, J., and LENDRUM, A. C. **Further observations on the use of synthetic resin as a substitute for Canada balsam.** *J. Path. & Bact.*, **53**, 441–3. 1941.

The polystyrene mounting medium previously described (abs. in Stain Techn., **15**, 77, 1940; *cf.* Hutner, Stain Techn., **16**, 177, 1941) is improved by replacing the tricresyl phosphate plasticiser with dibutyl phthalate as follows: mix 5 cc. of dibutyl phthalate with 35 cc. of xylene; dissolve 10 g. of polystyrene ("Distrene 80") in the mixture. The new medium is less tacky at the edge of the coverslip. Preparations with the newer medium have not deteriorated after 14 months. The lapse of time since the earlier publication has afforded further proof that delicate stains, such as Romanowski mixtures, are well preserved. In one uncontrolled series there was fading of hemalum-eosin, which was not observed in other slides so stained. To avoid precipitation of paraffin in the old or new medium, preparations should be mounted from fresh xylene.—*S. H. Hutner.*

DYES AND THEIR BIOLOGICAL USES

HENDERSON, H. J., and LONG, E. R. **Effect of chlorin-e-rhodin-g on experimental tuberculosis.** *Proc. Soc. Exp. Biol. & Med.*, **48**, 435–7. 1941.

Chlorin-e and rhodin-g, substances which are produced when chlorophyll is broken down, together inhibit the growth of tubercle bacilli *in vitro*. They do not affect the course of experimental tuberculosis in rabbits. The pigment accumulates in large quantity in phagocytic cells, in spleen, and in bone marrow. Pigmented cells frequently are concentrated on the border of tubercles.—*M. S. Marshall.*

MOORE, MAURICE L., and MILLER, CHARLES S. **Sulfonamidothiazolones.** *J. Amer. Chem. Soc.*, **63**, 2781. 1941.

The preparation and properties of a number of sulfonamidothiazolones are described. Sixteen 2-sulfanilamido-4-thiazolones have been tested for their chemotherapeutic effect against experimental streptococcal and pneumococcal infections in a standard stock strain of white mice, and show promising activity. The antistreptococcal activity was determined by the oral administration of daily doses of 5 mg. for four days to 20-g. mice experimentally infected with 1000 M.L.D. of a virulent strain of β-hemolytic streptococcus. The initial dose was given at the same time as the intraperitoneal injection of the infective culture. The antipneumoccoccal activity was determined by the oral administration of 20 mg. doses to mice infected with 100 M.L.D. of type I pneumococci at the time of the first dose of compound. Five additional doses were administered at 7, 24, 48, 72 and 96 hr.

In order to correlate the chemotherapeutic effect of these compounds, the relative absorption and blood concentration of 2-sulfanilamido-5-ethyl-4-thiazolone for comparison with sulfanilamide, sulfapyridine and sulfathiazole was determined in mice at a dosage of 0.25 g. and 1.0 g. per Kg. of body weight. It was found that 2-sulfanilamido-5-ethyl-4-thiazole was absorbed as rapidly and its maximum concentration was higher on both levels than for the other three compounds. It also appeared to be completely eliminated from the blood between 8 and 24 hr. after administration at both dose levels.—*A. P. Bradshaw.*

RAIZISS, GEORGE W., and CLEMENCE, LEROY W. **2-Sulfanilyl-aminothiazoline.** *J. Amer. Chem. Soc.*, **63**, 3125. 1941.

The preparation and chemical properties of 2-sulfanilyl-aminothiazoline (sulfathiazoline) and related compounds are described. In tests on various species of animals, sulfathiazoline was found to be of low toxicity. When given by mouth it is absorbed quickly into the blood stream. The therapeutic effect of this compound in mice with types II and III pneumococcus infection was about the same as observed with sulfathiazole. In *Staphylococcus aureus* infection, sulfathiazoline proved to be superior to sulfathiazole.—*A. P. Bradshaw.*

SPRAGUE, J. M., KISSINGER, L. W., and LINCOLN, R. M. **Sulfonamido derivatives of pyrimidines.** *J. Amer. Chem. Soc.*, **63**, 3028. 1941.

The substitution of pyridyl and thiazolyl radicals into the *N* position of sulfanilamide has resulted in compounds of improved therapeutic value. In continuing the study of heterocyclic derivatives of sulfanilamide, a number of sulfonamidopyrimidines, in general, exhibit a high order of activity in experimental infection.—*A. P. Bradshaw.*

WRIST, HAROLD, and JENKINS, GLENN L. **Heterocyclic derivatives related to sulfanilamide. I. The quinoline analog of sulfanilamide and derivatives.** *J. Amer. Chem. Soc.*, **63**, 2943. 1941.

The successful use of sulfanilamide and its derivatives in chemotherapy made it desirable to prepare quinoline analogs of two basic types, namely, 5-aminoquinoline-8-sulfonamide and 8-aminoquinoline-5-sulfonamide. Two other related compounds were prepared: 5-nitro-*N*-(2-pyridyl)-8-quinolinesulfonamide and 5-nitro-*N*-(2-thiazyl)-8-quinoline sulfonamide. The compounds are being tested pharmacologically and bacteriologically.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

BRAND, E. **Über eine brauchbare Gegenfärbung zur Holzerschen Darstellungsmethode der faserigen Neuroglia.** *Zts. gesam. Neurol. U. Phychiat.*, **172**, 531–5. 1941.

The author proposes a picro-fuchsin counterstain for the Holzer procedure in staining neuroglia. The technic recommended is:

Prepare 10–15 mμ frozen sections of formalin-fixed nervous tissue and stain by Holzer's method as given in Spielmeyer's *Technik* (1930), but differentiate less than usual. Flush the slide with xylene, blot with paper; absolute alcohol, blot; 95% alcohol, blot. Work rapidly. Apply several drops of the picrofuchsin stain, 10–15 sec., pour off and blot. (The picrofuchsin is made by mixing 7 parts of saturated aqueous picric acid with 5 parts of 1% aqueous acid fuchsin, when used for brain; or 2 parts of the former to 1 part of the latter, when used for spinal cord.) Apply absolute alcohol or better still, isopropyl alcohol, then two applications of xylene, blotting the slide after each. Cover in balsam.

Glia fibers stain blue or blue-black with a violet cast against a contrasting ground. The method gives improved differentiating coloration between fibrillar and cytoplasmic structures especially in pathologic gliosis.—*H. A. Davenport.*

DE LA FUENTA, VICTORINA. **A new method of staining with Wright's solution.** *J. Lab. & Clin. Med.*, **27**, 351–2. 1941.

The following technic is simple, rapid, and when applied to a blood film, reveals the structures clearly. Cover the air-dried smear with a few drops of Wright's

stain. Blow on the smear with the breath until it becomes pinkish-violet in color (about 10 sec.) Wash with tap water. With tap water running slowly over the slightly tilted slide, pour on a few drops of Wright's stain quickly. Wash, dry and examine with the oil immersion lens. Blowing the smear accelerates staining from minutes to seconds. Washing with the stain itself dissolves any precipitated particles of stain.—*John T. Myers.*

HEATH, JAMES P. **The nervous system of the kelp crab, Pugettia producta.** *J. Morph.*, **69**, 481–500. 1941.

Methylene blue failed to differentiate the nervous system well in this species. Of the several stains used, toluidine blue proved to be most satisfactory, since it usually gave a strong coloration to the nerve sheaths.

The author did not indicate the sources of the dyes, their certification numbers, concentration of solutions, or methods of application.—*Elbert C. Cole.*

FRENCH, C. **A combined stain for fat and elastic tissue.** *Arch. Path.*, **30**, 1243. 1940.

For simultaneous staining of fat and elastic fibers, the author recommends the following method of embedding: Fix tissues in a solution of formaldehyde (strength not given) for 24 hr. or longer. Wash in running water for 5 hr. Place in 5% aqueous solution of gelatin (Knox) at 37° C. for 12–24 hr.; in 10% gelatin at 37° C. for 6–12 hr. Embed in 10% gelatin. Harden in refrigerator for 2–4 hr. Before cutting, place individual blocks in formaldehyde solution for at least 12–24 hr. Cut sections on freezing microtome at 10 μ. Before staining, place sections in formaldehyde for 12–24 hr.

The staining procedure recommended is: Rinse section well in distilled water. Dip several times in 70% alcohol. Place for 15 min. in the following fat and elastic tissue stain, warmed to not more than 45° C.: Weigert's elastic tissue stain (ripened), 10 ml.; distilled water, 2 ml.; saturated solution of "scarlet red" (presumably Sudan IV) in equal parts of acetone and 70% alcohol, 8 ml. Rinse in 70% alcohol; then in distilled water. Place in Delafield's (or other) hematoxylin for 1–2 min. Rinse in distilled water. Differentiate in 0.5% acid alcohol, if desired. Immerse in weak ammonia until the section becomes blue. Rinse in distilled water. Transfer to a slide, drain, and mount in glythrogel. (*From J. Lab. and Clin. Med.*, **26**, 1991–2.)

KAY, W. W., and WHITEHEAD, RAYMOND. **The role of impurities and mixtures of isomers in the staining of fat by commercial Sudans.** *J. Path. & Bact.*, **53**, 279–84. 1941.

A study of many dyes of the Sudan III and IV classes indicated that mixtures of purified Sudans stained more strongly than the individual components, and that commercial impure Sudans stained better than the pure Sudans. Sudan IV (British Drug Houses No. 555722) was the most satisfactory.—*S. H. Hutner.*

KRAJIAN, A. **A rapid method of staining fat in frozen sections with osmic acid.** *Arch. Path.*, **30**, 766. 1940.

The following procedure is recommended for staining fat: Fix tissue in 10% formaldehyde for 24 hr. Cut frozen sections 10 μ thick. Bring to a boil a 1% aqueous osmic acid in a Pyrex test tube and pour into a small Stender dish. Place the section in the hot osmic acid solution and keep in a paraffin oven at 60° C. for 5 min. Wash in a basin of tap water. Counterstain in 1% aqueous eosin or phloxine for 1 min. Wash rapidly in tap water. Transfer to a slide and allow to drain. Mount in 3 large drops of glycerin jelly (previously melted in paraffin oven or water bath). Results: The fat globules are black or gray black; the background is red.

For emergency examination, bring a 10% solution of formaldehyde to boil in a Pyrex test tube (60 ml. capacity); drop a thin piece of biopsy or autopsy material into it, and place the specimen in an oven at 60–65° C. for 10 min. Cut thin frozen sections and stain by the method described.

It is not necessary to discard the used osmic acid solution. It may be poured back into the stock bottle and used over and over.—(*From J. Lab. and Clin. Med.*, **26**, 1991.)

PEERS, J. H. **A Modification of Mallory's phosphotungstic-acid-hematoxylin stain for formaldehyde-fixed tissues.** *Arch. Path.*, **32**, 446. 1941.

After formaldehyde fixation the author recommends the following schedule: Deparaffinize sections and bring down to water. Mordant 3 hr. in a saturated solution of $HgCl_2$ in a paraffin oven at 57° C. Rinse briefly. (The $HgCl_2$ solution may be used repeatedly.) Place in "compound solution of iodine" (i.e. Lugol's solution) for 5 min. Rinse. Place in 5% aqueous $Na_2S_2O_3$ for 5 min. Rinse. Place in 0.25% $KMnO_4$ for 5 min. Rinse. Place in 5% aqueous oxalic acid for 5 min. Wash well. Stain overnight in phosphotungstic acid hematoxylin. Wash briefly in tap water, dehydrate in alcohol or acetone, clear in xylene and mount in balsam.

The technic gives results almost identical with those of the original method as applied to tissue fixed in Zenker's solution. Neuroglia, fibroglia, and myoglia fibrils, belpharoplasts, nuclei, red blood cells, and fibrin are deep blue, while collagen is brownish red. (*From J. Lab. & Clin. Med.*, **27**, 701.)

SAZERAC, R., and POUZERGUES, J. **Recherche du bismuth dans les cellules et les tissus animaux. Formation de cristaux caractéristiques.** *Ann. Inst. Pasteur*, **66**, 90–5. 1941.

To demonstrate phagocytized bismuth in smears or sections, the following fresh aqueous mixture is prepared: 2% *o*-hydroxyquinoline, 1 cc.; 4% KI, 1 cc.; 3% HNO_3, 8 cc. The mixture forms an orange-red double salt with bismuth up to a dilution of 1:1,000,000. After intraperitoneal injection of an aqueous suspension of bismuth, proceed as follows: Smear the peritoneal fluid, dry, and fix with heat. Add 1 drop of fresh reagent, then a coverslip. The phagocytes are colored yellow, grains of bismuth orange-red, and the crystals grow from these granules to resemble the sheaves of prisms of certain osazones. Section tissues as usual after formalin fixation, and add the reagent after removal of paraffin. Crystallization is favored by heating the slide at 80° for 2–3 min.—*S. H. Hutner.*

ZAHL, P. A., and WATERS, L. L. **Localization of colloidal dyes in animal tumors.** *Proc. Soc. Exp. Biol. & Med.*, **48**, 304–10. 1941.

Evans blue, lithium carmine and nine other dyes were injected intravenously into tumor-bearing mice. Tumors were studied histologically. Acid dyes did not localize in the tumor cells but in the surrounding stroma. Further localization took place within the tumor at the interface between viable and necrotic tissue. Small repeated doses did not localize in greater amounts than a single injection given in the same amount. There was a difference in behavior between colloidal dyes and colloidal carbon.—*M. S. Marshall.*

PLANT MICROTECHNIC

BALL, ERNEST. **Microtechnique for the shoot apex.** *Amer. J. Bot.*, **28**, 233–43. 1941.

The author describes a series of improved methods, from fixation to sectioning, for preparing sections of shoot apices of vascular plants. Chamberlain's formula (5 cc. glacial acetic acid; 5 cc. commercial formalin, and sufficient 70% ethyl alcohol to make 100 cc. of solution) is modified by replacing the ethyl alcohol with the same volume of methyl, iso-propyl, *n*-propyl or tri-butyl alcohol, and the acetic acid with an equivalent amount of another fatty acid, e.g. 3.7 cc. formic (88% sol.), 6.5 cc. propionic, 8 cc. butyric, or 9.4 cc. valeric. Similar modifications of Juel's solution and of several aqueous fixatives are also tried. Fixation is for 24 hr., followed by washing in a 70% solution of the corresponding alcohol. A method for dehydratifig tissues in alcohol over CaO and $CuSO_4$ is also described.

Only ethyl alcohol was found to give good results when used in the fixing solutions. Acetic acid was found to be the most suitable of the fatty acids in preparing fixatives. Several phases of the effects of different fluids used in the preparation of sections of shoot apices for microscopic examination are discussed.—*T. M. McMillion.*

MICROÖRGANISMS

AMBROSIONI, P. **Modificazione del metodo di Gins per la colorazione del B. difterico.** *Ann. Igiene*, 50, 228–30. 1940.

The author's modification of the Gins method is simple and is claimed to be more efficient than the original. The procedure: After fixing smear in ordinary fashion, let the first stain (1 g. crystal violet, 15 cc. glacial acetic acid, 285 cc. distilled water) act for 10–15 sec. Wash. Apply second stain (chrysoidin, 2 g. in 100 cc. distilled water) for 20–30 sec. Wash. Mordant (Lugol's solution 99 cc., lactic acid 1 cc.) 5 sec. Wash. Dry. Examine.—*Carl Lamanna.*

FLEMING, ALEXANDER. **Some uses of nigrosin in bacteriology.** *J. Path. & Bact.*, 53, 293–6. 1941.

The following solution is recommended for negative staining of bacteria: nigrosin, water-soluble (Gurr), 10 g.; water, 90 cc.; formalin, 10 cc. The nigrosin may be mixed with the bacteria and spread either like a blood smear or with a loop. Stained bacteria can be overspread with nigrosin to demonstrate capsules in the following manner: As nigrosin solution is a decolorizer, dry slides rapidly. To combine spore-staining and negative staining, stain spores the usual way with carbol fuchsin, rinse off excess dye with water and alcohol. Cover with the nigrosin solution 5 min. or longer, wash off with tap water. Place a drop of nigrosin on one end of the slide, spread with another slide over the stained portion and allow to dry. Bright red spores show against the unstained bodies. (This latter is a slight modification of the method described by Dorner in 1922, but no credit is given.)—*S. H. Hutner.*

MANOUÉLIAN, Y. **Variations dans l'argyrophilie des spirochètes.** *Ann. Inst. Pasteur*, 66, 83–9. 1941.

Faintly stained or unstained spirochetes are not uncommon in tissues known to be infectious. Tissues kept in formalin for 10–15 years may be impregnated perfectly; yet sometimes impregnation, for unknown reasons, may fail both in fresh and old tissues. Absence of morphologically demonstrable spirochetes in infectious tissues should therefore not be ascribed to a filterable stage in the life history of the spirochete.—*S. H. Hutner.*

PIJPER, ADRIANUS. **Dark-ground studies of Vi agglutination of B. typhosus.** *J. Path. & Bact.*, 53, 431–6. 1941.

This continuation of the author's studies on bacterial agglutination (abs. in Stain Techn., 14, 73, 1939) is illustrated with remarkable photomicrographs of H, O and Vi agglutination, which clearly show the role of flagella and of the remainder of the cell in these morphologically distinct types of agglutination. Three 16-mm. motion picture films have been prepared by the author illustrating (1) sunlight dark-field technic and motility of typhoid bacteria, (2) O aggutination and (3) H agglutination of typhoid bacilli. A film on Vi agglutination is in preparation.—*S. H. Hutner.*

PRYCE, D. M. **Sputum film cultures of tubercle bacilli: a method for the early observation of growth.** *J. Path. & Bact.*, 53, 327–34. 1941.

Micro-culture of tubercle bacilli offers a possibility of more rapid diagnosis. Other microbes on a sputum smear are destroyed by acid, the smear is then incubated in contact with a clear culture medium, and the microcolonies of tubercle bacilli are stained so as to be visible with low powers of the microscope. Two methods are described in detail: (1) Spread sputum on the flat or slightly concave bottom of a 4 in. Petri dish. Treat the dried film with 15% H_2SO_4 for 5 min., pipette off the acid, flood the smear with 5 cc. of distilled water and allow to act for about 1 min. Repeat the washing 1–2 times. Add the medium (prepared by laking citrated human blood with an equal volume of 1% saponin) and incubate 1 week. Wash off the medium with water and stain with Ziehl-Neelsen as usual. Counterstaining of the colonies is unnecessary. (2) Smear and dry one or more circular patches of sputum on a glass slide. Immerse in melted vaseline a bakelite, hard fiber, or glass ring about 1–2.5 cm. diameter. Drain off excess vaseline and apply the ring about a patch of sputum. Apply acid and rinse as before, then incubate in contact with the medium either in a moist

chamber made from a Petri dish or on a slide closed with a vaselined coverslip. Stain as before.

Notes: Rings may be cut from tubing, then sandpapered. Vaseline does not interfere with staining if the stain is rewarmed before rinsing. All solutions used must be sterile. If a closed chamber is used for incubation, the blood should be well oxygenated and adequate in amount. Besides killing non-acid-fast organisms, the acid coagulates and "fixes" the mucus; thick layers of mucus may be evaporated. Laryngeal swabbings give better results than sputa. The technic can be applied to pus and caseous material.—*S. H. Hutner.*

SHERIDAN, B. W. **A rapid method of dehydrating and clearing iron-hematoxylin-stained fecal smears.** *J. Lab. & Clin. Med.*, **27**, 254. 1941.

The following method shortens the preparation of wet-fixed stained smears for the identification of intestinal protozoa. Fix in Schaudin's solution (with glacial acetic acid added up to 5%) for 5 min. Place serially in: tap water, 3 min.; 95% alcohol (with iodine added to make a port wine color), 3 min.; 4% $FeNH_4(SO_4)_2$, 15 min.; rinse in tap water; apply 0.5% aqueous hematoxylin, 10 min.; 0.25% $FeNH_4(SO_4)_2$, 10 min.; running water, 1 min.; cellosolve (ethylene glycol monoethyl ether), 10 min.; mount in balsam. This method is quick, satisfactory and as permanent as any other technic.—*John T. Myers.*

HISTOCHEMISTRY

HAMAZAKI, Y. **Über den Chemismus des Modellversuches für die Karbolfuchsin-Jod-Methode (eine neue Nachweismethode für Nucleinsaure und Purinderivate).** *Trans. Soc. Path. Jap.*, **30**, 111–5. 1940.

After fixation in chromic acid solutions, animal (but not plant) nucleic acids are stained by a procedure which appears to combine features of the Fuelgen and Gram technics. Details of the staining procedure are not given, but the staining solution appears to consist of a mixture of Lugol's iodine and basic fuchsin. Staining is followed by decolorization with a strongly acid solution of $NaHSO_3$. The difference between plant and animal nucleic acids is ascribed to the presence of desoxyribose in animal nucleic acid and ribose in plant nucleic acid. Oxidation of the desoxyribose by the CrO_3 creates a keto-enol group with which the iodine and fuchsin combine, and which resists decolorization. Oxidation of ribose does not create the necessary keto-enol group. This theory is based on the formation of a similar purple dye complex from desoxycholic acid but not from cholic acid. Treatment of cholic acid with CrO_3 renders it stainable. Since the procedure is considered a test for keto-enol groups, it is pointed out that products of nucleic acid hydrolysis, such as adenine and guanine, which on CrO_3 oxidation yield products containing the keto-enol group, also stain purple.—*S. H. Hutner.*

KNAYSI, GEORGES. **On the use of basic dyes for the demonstration of the hydrolysis of fat.** *J. Bact.*, **42**, 587–9. 1941.

Several basic dyes other than Nile blue can be used to demonstrate the hydrolysis of fats by microörganisms if the dyes are converted to their free bases, by the following procedure: Liberate the free base with *N* NaOH added drop by drop until precipitation is complete; filter; wash the precipitate with distilled water adjusted to about pH 7.5; dry and store the base; or use it to saturate the fat incorporated with the culture medium, which is then sterilized by autoclaving. Nile blue, neutral red, methylene blue, and malachite green have been tested; the colors of the neutral fat are red, orange-yellow, red, and olive green respectively; the corresponding colors of the free fatty acids are blue, red, blue and blue-green. Any basic dye with the color of the free base differing from that of the salt could be used. The medium in which the colored fats are used should be neutral or slightly alkaline, highly buffered, and should not contain fermentable sugars because acids in the aqueous phase extract the dye from the fat forming a dye salt in the medium and leaving the fat drops uncolored.

Nile blue is often toxic, but this may be overcome by using it as follows: Shake 1 ml. of a saturated aqueous solution of its sulfate or chloride with 10 ml. neutral fat, separate the red fat from the blue solution in a separatory funnel, wash with distilled water, and sterilize by autoclaving. One ml. of this fat shaken with 100 ml. of agar medium is used for plating.—*Virgene Kavanagh.*

STAIN TECHNOLOGY

VOLUME 17 JULY, 1942 NUMBER 3

STUDIES ON POLYCHROME METHYLENE BLUE

II. Acid Oxidation Methods of Polychroming

R. D. LILLIE, *Senior Surgeon, Division of Pathology, National Institute of Health, Bethesda, Maryland*

ABSTRACT.—The action of $K_2Cr_2O_7$, Ag_2O, $KMnO_4$, HgO and $NaIO_3$ in polychroming methylene blue is explored. The last two have no action in neutral or acid methylene blue solutions. With the other three reagents the amount of polychroming, as measured by the shift in the absorption spectrum, is roughly proportional to the amount of oxidant used. Various lots of methylene blue produce similar products with similar proportions of $K_2Cr_2O_7$. With similar quantities of this reagent similar products are produced by polychroming at 100°, 80°, 70° or 60° C. At 100° C. the action of $K_2Cr_2O_7$ or of Ag_2O appears to be completed in 15 minutes. In $K_2Cr_2O_7$ polychroming, H_2SO_4 can be substituted for HCl, and subsequent $BaCO_3$ neutralization removes the salts formed and prevents accidental alkali polychroming. $K_2Cr_2O_7$ polychroming produces products with narrower absorption bands than alkali polychroming.

Having shown the importance of azure B and its eosinate in the staining of malaria parasites (Roe *et al* 1940, 1941) and correlated the staining performance for blood and plasmodia of various Romanowsky stains with their absorption spectra (Lillie *et al* 1942), it was decided to try out the acid chromate process of oxidizing methylene blue as a part of a more comprehensive study. This method has been used by MacNeal, (1906, 1925), by Scott and French, (1924), by Holmes and French (1926). These writers had been more interested in the production of azure A, azure C and methylene violet than in azure B, a dye which they considered of little value.

Starting from the results of Holmes and French, it was decided to try HCl-$K_2Cr_2O_7$ oxidations with lesser quantities of $K_2Cr_2O_7$ than they used, to see whether or not polychrome methylene blue could be produced which consisted mainly of azure B.

Hence, 5-g. portions of methylene blue (E & A)[1] were boiled 45 minutes under a reflux condenser in 300 cc. distilled water and 10 cc. concentrated (38%) HCl, adding just at the start of boiling a variable amount of 10% aqueous $K_2Cr_2O_7$ solution. At the end of the heating 1 cc. samples were removed for spectral analysis and half the sample neutralized to nitrazine paper (pH 6.8) with saturated Na_2CO_3 solution. Then the eosinate was precipitated by the addition of 23 cc. 10% eosin Y (LE-11). The eosinates were filtered out next morning using a vacuum filter, dried and dissolved in the proportion of 0.1 g. to 80 cc. C.P. methanol.

These solutions were tested on blood films containing *Plasmodium malariae*, as in the previous paper, and the results are given in the last two columns of Table 1. As noted in the earlier paper, best staining of the plasmodia occurred when polychroming of the methylene blue had proceeded to such an extent as to give an absorption maximum between 660 and 640 mμ, indicating a high proportion of azure B. In the present experiment, when the absorption maximum of the polychromed methylene blue (column 4) is taken into account it will be seen that the shift of the absorption maximum and the widening of the band, at which D was over 90%, was strictly in the order expected from the amount of $K_2Cr_2O_7$ used. Staining performance did not follow this order, however, as there were a few instances where the staining of the plasmodia was as poor as in the case of sample I-K, the unpolychromed dye—notably I-H and I-I, and to a less extent I-E and I-G, which showed the gray lilac staining of the parasite cytoplasm and the redder chromatin which would be expected of azure A stains, rather than the azure B picture which would have been expected from the spectra of the thiazin. In an effort to explain this discrepancy, absorption spectra of the thiazin eosinates were made; and comparison of these spectra with those of the freshly polychromed, still acid, methylene blue, revealed that these four samples had developed considerable shifts of their thiazin absorption spectra in the process of being converted into eosinates. This comparison further revealed that samples I-A and I-B, which unexpectedly gave better staining than sample I-C, had also undergone further polychroming in the process of preparation of the eosinate, whereas sample I-C had not.

As the differences between the first series of polychrome methylene blues and their eosinates were attributed to alkali polychroming by the Na_2CO_3 used in neutralization, it was determined to use an insoluble carbonate for neutralization. As it seemed desirable at the

[1]E & A, procured from Eimer and Amend before 1925.

TABLE 1. SPECTRA OF POLYCHROME METHYLENE BLUE SAMPLES AND THEIR EOSINATES WITH THE STAINING PERFORMANCE OF THE LATTER. HCl, $K_2Cr_2O_7$ SERIES

Lot No.	Graph No.	Mg. $K_2Cr_2O_7$ per g. M. B.	Thiazin			Thiazin eosinate				Staining performance†	
			λ	90% D λ band*		Eosin λ	Thiazin λ	90% D λ band of thiazin			
				Width	Median			Width	Median	Blood	Plasmodia
I-K	1, 68	0.0	664.5	18.3	662.3	516	663	18.3	661.1	±	±
I-A	2, 75	20	663.5	19.6	662.0	517	654	36.9	649.0	+++	+++
I-B	3, 76	40	663.5	19.1	662.0	516	654	35.1	653.9	+++	++
I-C	4, 77	60	662.0	21.5	660.0	517	662	24.0	660.1	+	±
I-D	5, 78	100	659.0	22.8	657.7	517	650	26.3	649.5	+++	+++
II-A	6, 158	100	658.0	23.0	656.9	516	658	23.1	656.7	+++	+++
II-B	159	120				516	657	25.7	655.7	+++	+++
II-C	160	140				516	657	26.3	654.9	+++	++
I-E	7, 79	150	655.5	24.3	654.3	517	635	40.2	637.5	+++	++
II-E	8	160	655.5	25.4	654.7	516	656	25.7	655.5	+++	++
II-F		180				516	657	26.8	656.2	+++	++
I-F	9, 80	200	654.0	26.4	651.8	517	642	37.6	640.6	+++	+++
II-G		200				516	658	25.5	656.9	+++	++
I-G	10, 81	250	651.0	31.0	647.0	517	635	35.4	633.7	+++	++
I-H	11, 82	300	649.0	31.8	646.3	517	625	33.7	624.2	+++	+
I-I	12, 83	400	642.0	44.1	636.7	517	617	30.6	616.1	+++	±

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.
†In this column symbols signify as follows: ±, poor; +, fair; ++, good; +++, excellent.

same time to attempt to eliminate the additional salt content produced by introduction of acid and its subsequent neutralization, H_2SO_4 was substituted for HCl and $BaCO_3$ for Na_2CO_3. Calculation showed that 3.4 cc. concentrated H_2SO_4 (sp. gr. 1.835) was approximately equivalent to 10 cc. 38% HCl, and that this would be neutralized by about 11.75 g. $BaCO_3$.

Four 5-g. samples of methylene blue (E & A) were dissolved each in 300 cc. boiling distilled water containing 3.4 cc. concentrated H_2SO_4, with 7.5 cc. 10% $K_2Cr_2O_7$ added to each. These were boiled respectively 45 minutes, 1½ hours, 3 hours and 6 hours under a reflux condenser. After adding 18 g. $BaCO_3$ (a 50% excess over the theoretical amount), boiling was continued another 20 minutes. The dye solution was then filtered on a vacuum filter and the barium salts washed with several small washes of hot water, bringing the total volume to 501 cc. One-cubic-centimeter samples were taken for spectral analysis and half of each sample was precipitated with 23 cc. 10% eosin Y (LE-11) as before.

Table 2 reveals that the thiazins alone and as eosinates give essentially identical spectra and that the difference in heating time has made no appreciable difference in the product.

As the foregoing experiments had not adequately explored the azure A range in acid bichromate polychroming, nor the minimum time for completion of oxidation, and as it appeared at that time that polychroming at lower temperatures by this process did not carry the demethylation beyond azure B, an experiment was set up to fill in these deficiencies. In the determination of the time requirements spectrophotometrographic examinations were made first of the samples heated the shortest times, until no further alteration was evident; and to check on this, the sample heated the longest time was examined. While intervening samples were taken, their examination was deemed unnecessary if samples heated for longer and shorter periods were essentially identical.

Examination of the data in Table 3 indicates that at 97°C. the oxidation is virtually completed in 10 minutes and fully so in 15 minutes, while no further change occurs with 1 and 4 hours heating. One striking thing is the narrowness of the absorption bands produced by the acid oxidation process in the 620–630 mμ area as compared with those produced by the alkali processes. Substantially the same grade of change is produced by heating with 500 mg. $K_2Cr_2O_7$ per gram methylene blue for any of the following periods and temperatures: 15 minutes at 97°C.; 1 day or 1 hour at 80°C.; 8 hours at 70°C.; 1 day (or less) at 60°C. More than 500 mg. $K_2Cr_2O_7$ per gram methylene blue produces little further effect.

TABLE 2. POLYCHROME METHYLENE BLUES AND THEIR EOSINATES, VARIATION OF HEATING TIME, H_2SO_4-$K_2Cr_2O_7$ SERIES, $BaCO_3$ NEUTRALIZATION

Lot No.	Graph No.	Mg. $K_2Cr_2O_7$ per g. M. B.	Time heated	Thiazin			Thiazin eosinate			
				λ	90% D λ band*		Eosin λ	Thiazin λ	90% D λ band of thiazin	
					Width	Median			Width	Median
IV-AB	22, 92	150	45 min.	655	25.1	653.1	517	655	27.9	652.8
IV-CD	23, 93	150	90 min.	655	24.1	653.9	517	655	28.3	652.6
IV-EF	24, 94	150	3 hours	655	25.2	653.5	517	654+	28.5	653.9
IV-GH	25, 95	150	6 hours	655	23.7	653.6	517	653	27.7	651.3

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

TABLE 3. SPECTROPHOTOMETRIC DATA ON METHYLENE BLUE SAMPLES HEATED IN 1.1% H_2SO_4 SOLUTION IN 1% CONCENTRATION WITH VARYING AMOUNTS OF $K_2Cr_2O_7$, FOR VARYING LENGTHS OF TIME AND AT 97°C., 80°C., 70°C. AND 60°C.

Graph No.	Dur. of heating	Temperature	$K_2Cr_2O_7$ per gram methylene blue	Absorpt. Maximum	90% D λ band data*	
					Width	Median
361	1 hr.	97°C.	0 mg.	662	18.8	660.5
362	"	"	50 mg.	660	19.8	659.0
363	"	"	100 mg.	658	19.0	657.7
364	"	"	150 mg.	655	23.0	654.15
365	"	"	200 mg.	650	26.4	648.1
366	"	"	300 mg.	644	32.5	641.0
367	"	"	400 mg.	634	31.5	633.2
368	"	"	500 mg.	628	29.4	627.3
369	"	"	750 mg.	625	24.3	623.9
370	"	"	1000 mg.	622	23.7	621.6
371	5 min.	"	500 mg.	632	33.6	631.5
372	10 min.	"	500 mg.	630	28.0	629.0
373	15 min.	"	500 mg.	628	28.3	626.7
374	4 hr.	"	500 mg.	629	30.3	627.7
376	1 hr.	80°C.	500 mg.	631	29.3	630.3
377	1 day	"	500 mg.	629	28.0	628.6
378	8 hr.	70°C.	500 mg.	628	28.2	627.2
379	4 days	"	500 mg.	628	33.6	627.1
380	1 day	60°C.	500 mg.	629	29.4	628.3

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.

λ is the wave length at which maximum absorption occurs.

The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

All the foregoing acid bichromate polychroming experiments were done with a single lot of methylene blue (E & A). Hence it seemed necessary to repeat sufficient of them on various other lots of methylene blue to show the general applicability of the method.

Therefore, 1-g. lots of various methylene, ethylene and thionine blues were dissolved each in 61.7 cc. of distilled water containing 1.13 cc. concentrated H_2SO_4 (sp. gr. 1.84). One cubic centimeter was taken from each lot for spectrophotometric examination, and each lot was then divided into 5 portions of 12 cc. each, to which were added severally 1.0, 1.25, 1.5, 1.75 and 2.0 cc. of 2% aqueous $K_2Cr_2O_7$, equivalent respectively to 100, 125, 150, 175 and 200 mg. $K_2Cr_2O_7$ per g. methylene blue. These were heated simultaneously in test tubes in a bath of boiling water for 90 minutes and removed from the water bath. To each tube 0.6 g. $BaCO_3$ was added, and heating was continued for another 30 minutes. The various samples were then filtered and stored in labelled vials for spectrophotometric study. The results of these studies are shown in Table 4.

It appears that the amount of demethylation is quantitatively quite similar for the various amounts of bichromate with the modern American samples: the two lots of LA-7, the NA-8 and the uncertified lot from Hartman-Leddon Co. The sample from the H. S. Laboratory, labelled thionine blue appears to resemble more the modern American methylene blues. The old Grübler 12.13 (probably 1913) sample shows quantitatively greater demethylation, in accord with its probably lower dye content. The Eimer and Amend lot labelled ethylene blue, which term is a synonym of methylene blue, does not appear to be that dye, but resembles more the sample of thionine blue (C.I. No. 926, not thionin C.I. No. 920) obtained from the Commission on Standardization of Biological Stains in its absorption maximum, but its demethylation is apparently limited rather than progressive as with thionine blue. (Thionine blue is described as ethyl trimethyl thionin, and the first dealkylation product would appear to be an ethyl dimethyl thionin rather than trimethyl thionin which is azure B.)

In the course of this study it seemed of interest to determine what products were produced in the Wilson[2] method of polychroming with Ag_2O at boiling temperatures. Since the Wilson procedure calls for removal of one-third after 30 minutes heating, another third after 60 minutes and the balance after 90 minutes, these three time intervals were used on separate portions. Ag_2O was used with 0.5% $NaHCO_3$, as in the Wilson procedure, and also without the carbonate. Each combination was run in duplicate at different times, making a total of 12 lots. The technic was as follows: To 1 g. $AgNO_3$ in 8 cc. distilled water, add 0.2 g. NaOH in 2 cc. distilled water; let settle 20

[2]The earliest reference to this method which the author has been able to locate is in Stitt's Practical Bacteriology, Blood Work and Parasitology, *5th Ed.* (Philadelphia, 1918), pp. 239–40.

TABLE 4. SPECTROPHOTOMETRIC DATA ON VARIOUS LOTS OF METHYLENE BLUE, ETHYLENE BLUE AND THIONIN BLUE, POLYCHROMED 90 MINUTES AT 97°C. BY THE H_2SO_4-$K_2Cr_2O_7$ PROCESS WITH $BaCO_3$ NEUTRALIZATION

	$K_2Cr_2O_7$ per g. dye in mg.	Methylene blue samples†					Ethylene blue†	Thionine blue†	
		Lot VI-A H. L. Co. LA-7	Lot VII-C H. L. Co. LA-7	Lot VI-B Grübler 12.13	Lot VI-D N. A. Co. NA-8	Lot VI-E H. L. Co.	Lot VI-C E. & A.	Lot VII-A Mfg. unknown	Lot VII-B H. S. Labs.
Absorption maximum (λ)	0	665.0	664.0	664.0	665.0	663.5	668.0	668.0	664.0
	100	665.0	659.0	659.0	660.0	659.0	662.0	663.0	659.0
	125	658.0	657.0	656.0	656.0	658.0	663.0	663.0	657.0
	150	656.0	655.5	653.0	655.5	656.0	662.0	661.0	656.0
	175	654.0	656.0	652.0	653.0	655.0	660.0	659.5	655.0
	200	653.0	654.0	650.0	653.0	653.0	660.0	657.0	652.0
90% D λ band width*	0	18.0	18.4	19.2	18.1	18.9	16.8	13.1	20.4
	100	19.4	24.1	24.5	22.4	22.8	21.4	19.6	22.8
	125	21.6	24.1	24.4	24.7	23.5	21.1	21.6	25.0
	150	24.3	24.3	26.5	25.5	24.7	22.5	22.9	25.4
	175	24.3	26.7	25.8	24.4	25.4	23.5	22.9	24.0
	200	27.4	24.7	26.4	25.8	26.0	22.0	26.9	25.9

Median of 90% D λ band	0	663.3	662.5	663.1	662.9	662.8	666.8	668.7	663.1
	100	663.2	657.5	656.3	658.1	658.1	659.0	662.3	657.5
	125	657.6	656.2	654.9	654.9	656.8	661.6	661.6	655.8
	150	655.4	655.1	651.8	653.8	655.0	660.0	659.2	653.9
	175	652.5	653.3	650.0	652.5	653.4	659.6	658.8	652.6
	200	652.2	651.9	648.2	651.3	650.3	659.3	656.0	650.1

TABLE 5. EFFECT OF OXIDATION WITH $KMnO_4$ AT 98°C. ON THE ABSORPTION SPECTRUM OF METHYLENE BLUE IN ACID SOLUTION

Graph No.	$KMnO_4$ per g. M. B.	Absorption Maximum	90% D λ band*		Dilution and D λ actual	Dilution to give D λ=1.000
			Width	Median		
384	250 mg.	660	20.0 mγ	659.5	2 γ/cc. .6861	2.9 γ/cc.
385	500 mg.	657	23.8	656.8	2.6 γ/cc. .5638	4.6 γ/cc.
386	750 mg.	657	26.6	656.3	3 γ/cc. .5560	5.4 γ/cc.
387	1000 mg.	654	29.2	650.3	4.4 γ/cc. .5376	8.2 γ/cc.
390	500+1000 mg.	648	34.6	643.8	28.5 γ/cc. .5560	51.3 γ/cc.
391	500+1000+500 mg.	614	32.9	614.5	100 γ/cc. .7012	142.6 γ/cc.

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.
†The abbreviations indicating manufacturers of these samples are as follows: H. L., Hartman-Leddon of Philadelphia; N. A., National Aniline; E. & A., Eimer and Amend; H. S. Labs., the H. S. Laboratories of Philadelphia. The sample of thionine blue of unknown manufacture was obtained from the laboratory of the Stain Commission at Washington, D. C. in 1940.

TABLE 6. SPECTROPHOTOMETRIC DATA ON EOSINATES OF METHYLENE BLUE POLYCHROMED WITH SILVER OXIDE

Graph No.	Lot No.	$NaHCO_3$ g.	$AgNO_3$ g.	M. B. g.	Temp. °C.	Time heated min.	Absorption maxima		90% D λ band of thiazin*	
							Eosin	Thiazin	Width	Median
191	IX-G	0.5	1.0	1.0	100	30	517	618	34.7	618.8
193	IX-H	0.5	1.0	1.0	100	30	516	616	37.6	619.5
195	IX-I	0.5	1.0	1.0	100	60	517	614	31.0	612.9
198	IX-J	0.5	1.0	1.0	100	60	517	614	31.0	614.3
201	IX-K	0.5	1.0	1.0	100	90	517	614	30.6	613.4
205	IX-L	0.5	1.0	1.0	100	90	517	615	32.9	616.6
181	IX-A	0	1.0	1.0	100	30	517	620	49.8–	628.4
182	IX-B	0	1.0	1.0	100	30	517	624	45.0	632.1
184	IX-C	0	1.0	1.0	100	60	516	621	44.4	628.7
185	IX-D	0	1.0	1.0	100	60	516	622	47.6	627.2
187	IX-E	0	1.0	1.0	100	90	517	620	37.6	621.8
189	IX-F	0	1.0	1.0	100	90	516	622	44.3	626.2
206	IX-M	0	1.0	1.0	100	15	516	622	43.0	623.7
207	IX-N	0	1.0	1.0	80	15	516	621	40.2	622.0
216	X-A	0	1.0	1.0	97	60	516	622	44.0	626.9

224	X-I	0	0.5	0.5	97	60	517	622	37.9	622.3
217	X-B	0	0.8	1.0	97	60	516	628	53.5−	635.5
225	X-K	0	0.4	0.5	97	60	517	621	38.8	623.4
218	X-C	0	0.6	1.0	97	60	517	650	54.9+	638.9
226	X-L	0	0.3	0.5	97	60	517	623	46.2	627.4
219	X-D	0	0.5	1.0	97	60	517	652	53.8	639.0
227	X-M	0	0.25	0.5	97	60	517	634	50.6	631.1
220	X-E	0	0.4	1.0	97	60	516	655	36.0	650.6
228	X-N	0	0.2	0.5	97	60	516	632	56.2	633.6
221	X-F	0	0.3	1.0	97	60	516	649	53.0	634.9
229	X-O	0	0.15	0.5	97	60	516	629	56.9	634.2
222	X-G	0	0.2	1.0	97	60	516	656	32.7+	651.8
230	X-P	0	0.1	0.5	97	60	516	655	36.6−	650.2
223	X-H	0	0.1	1.0	97	60	516	660	25.6	655.9
231	X-R	0	0.05	0.5	97	60	516	656	35.4	651.2

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

minutes; pipette off and discard supernatant fluid; wash 4 times by adding 100 cc. distilled water each time, letting settle 20 minutes each time as before, and pipetting off supernatant fluid. To the moist Ag_2O add 100 cc. 1% solution of methylene blue in distilled water or in 0.5% $NaHCO_3$. Boil 30 minutes, adding small quantities of distilled water frequently to maintain volume.

Spectrophotometric data are presented in Table 6.

The results of the polychroming for 30, 60 and 90 minutes without $NaHCO_3$ are quite similar in the six lots, and also in two additional lots heated 15 minutes at 100° and at 80°C., giving thiazin absorption maxima between 620 and 624 mμ, and 90% Dλ bands about 37 to 50 mμ in width with medians between 622 and 632 mμ. With both $NaHCO_3$ and Ag_2O the alteration was consistently greater, and quite similar in the six lots, giving thiazin absorption maxima between 614 and 618 mμ and 90% Dλ bands 30 to 37 mμ in width with medians between 612 and 620 mμ.

In view of this information, the Wilson procedure of combining products heated 30, 60, and 90 minutes seems superfluous, as the maximal effect of Ag_2O (without $NaHCO_3$) seems to be evident after only 15 minutes even at 80°C.

At this point the question came up whether Ag_2O might not act quantitatively as an oxidizing agent, in the same manner as does $K_2Cr_2O_7$ in acid solution. To determine this question a series of lots of polychrome methylene blue was made by heating simultaneously, for 1 hour in an Arnold steam sterilizer at about 97°C., 16 lots of 1% methylene blue with the Ag_2O prepared as before from variable amounts of $AgNO_3$. The data presented in the second half of Table 5 show more irregularity than similar series with $K_2Cr_2O_7$, but it is plain that the larger amounts of Ag_2O cause the larger shift in the absorption maximum, and the lesser, smaller displacements. Irregularities may be at least partly explained by losses of Ag_2O in the process of its preparation and washing.

At this point some further exploration of other oxidizing agents as to their action on methylene blue was deemed advisable. As $KMnO_4$ has been used[3], this substance was selected for trial. A preliminary lot was made by boiling 100 mg. methylene blue, 33 mg. $KMnO_4$ and 0.02 cc. concentrated HCl in 20 cc. water for 5 minutes. This gave an absorption maximum at 655–660 and a 90% Dλ band width of 21.5 mμ with median at 657.9.

As these results appeared encouraging, a series of 10 lots of 100 mg.

[3]I recall the statement in an old French hematology text, which I have been unable to find since.

methylene blue each in 10 cc. 0.1% H_2SO_4 were heated 1 hour in a bath of boiling water with the following amounts of $KMnO_4$: 0, 5, 10, 15, 20, 30, 40, 50, 75 and 100 mg. As only a slight shift in absorption spectra occurred even with the largest amount of $KMnO_4$, the tubes were reheated for 2 hours more which produced no further change, the 100 mg. $KMnO_4$ lot still showing an absorption maximum at 658 mμ (659 at 1 hour), and a 90% Dλ band 22 mμ wide (22.5 at 1 hour) with median at 657.6 (657.5 at 1 hour). As the use of 0.1% H_2SO_4 had been due to an error in calculation, it was decided to repeat this experiment using 1.1% H_2SO_4 as the equivalent of the 3.4 cc. to 300 cc. which was used in the previous $K_2Cr_2O_7$ experiments. Satisfactory samples were obtained using 250, 500, 750 and 1,000 mg. $KMnO_4$ per gram methylene blue, but when 2.5 g. per gram of the dye was used, a brown granular precipitate was promptly produced which failed to redissolve with several hours heating. Addition of another gram of $KMnO_4$ to the lot polychromed with 500 mg. per gram dye left a blue dye after evolution of gas had ceased; therefore, after an hour at 98°C., a sample was taken for absorption spectrum, and after adding 500 mg. $KMnO_4$, was heated for another hour. Then another sample was taken and a further heating with an additional 500 mg. $KMnO_4$ followed, leaving much brown precipitate and only a faint color in the fluid. (See Table 5.)

The dilution data indicate that in addition to demethylation of part of the dye, much of it is entirely destroyed. This is confirmed by the fact that immediate or fractional addition of 2.5 g. $KMnO_4$ to 1 g. dye leaves a black granular residue in a water white solution.

HgO appeared to be inert when used with methylene blue dissolved in 1.1% H_2SO_4 or in distilled water using as much as 1.2 g. HgO per g. methylene blue in each case. Were the oxygen in this compound available, the quantity used should have been sufficient to oxidize 2 methyl groups and to form azure A. Absorption maxima remained at 662 as in the untreated methylene blue. $NaIO_3$, (480 mg. per gram methylene blue, enough theoretically to oxidize 2 methyl groups) was also inert.

Summary

Various lots of methylene blue, when heated with similar quantities of $K_2Cr_2O_7$ in acid solution yield closely similar products, and larger amounts produce quantitatively greater changes.

In polychroming with $K_2Cr_2O_7$ in acid solution the process appears to be completed in 15 minutes at 100°C. and no further change occurs with prolonged further heating. The same end product is formed with the same amount of bichromate whether the heating is at 100°,

80°, 70° or 60°C. More than 500 mg. $K_2Cr_2O_7$ per g. methylene blue produces little further effect at temperatures up to 100°C. The products generally present much narrower spectral absorption bands than do the products of alkali polychroming with similar absorption maxima. The use of H_2SO_4 in place of HCl, and subsequent $BaCO_3$ neutralization gives quite satisfactory results, avoids the possibility of further polychroming which may occur if Na_2CO_3 is used, and removes the mineral salts effectively from the final solution.

Ag_2O, when used alone, is similar to $K_2Cr_2O_7$ in its action, in that at 100°C. its action is completed in about 15 minutes and prolonged further heating gives no further alteration in the absorption spectrum, and further in that the amount of alteration produced is roughly proportional to the amount of Ag_2O used.

In 0.1% H_2SO_4 solution $KMnO_4$ produces little change in methylene blue, but in 1.1% H_2SO_4 polychroming occurs which is roughly proportional to the amount of $KMnO_4$ used. However, at the same time much of the dye is totally destroyed, prolonged oxidation with an excess resulting in a colorless solution and a black granular residue.

HgO and $NaIO_3$ produce no polychroming of methylene blue at 100°C. either in neutral or in acid solution.

REFERENCES

Holmes, W. C., and French, R. W. 1926. The oxidation products of methylene blue. Stain Techn., **1**, 17–26.

Lillie, R. D., and Roe, M. A. 1941. Studies on polychrome methylene blue. I. Eosinates, their spectra and staining. Stain Techn., **17**, 57–63.

MacNeal, W. J. 1906. Methylene violet and methylene azure. J. Inf. Dis., **3**, 412–33.

MacNeal, W. J. 1925. Methylene violet and methylene azure A and B. J. Inf. Dis., **36**, 538–46.

Roe, M. A., Lillie, R. D., and Wilcox, A. 1940. American azures in the preparation of satisfactory Giemsa stains for malaria parasites. Pub. Health Rep., **55**, 1272–8.

Roe, M. A., Wilcox, A., and Lillie, R. D. 1941. Eosinates of the azures and methylene blue in preparation of a satisfactory Giemsa stain from dyes of American manufacture. Pub. Health Rep., **56**, 1706–9.

Scott, R. E., and French, R. W. 1924. Standardization of biological stains. Milit. Surg., **55**, 229–43, 337–52.

THE MARCHI METHOD

FRED A. METTLER AND RUTH E. HANABA, *Department of Neurology College of Physicians and Surgeons, Columbia University, New York*

ABSTRACT.—**An investigation has been made of the poor penetration obtained by the Marchi method. Use of surface tension depressants or perfusion technics showed no marked improvement. Optimum results cannot be achieved with slices of tissue above 3 mm. in thickness. Another cause of faulty staining is the failure to maintain an adequate strength of OsO_4 in the Marchi mixture. The concentration of this chemical should never be allowed to drop below 0.25%. A chemical method for determining the amount of OsO_4 present in the staining mixture and based upon the Alvarez test is described. This method can also be utilized to reclaim partially exhausted staining solutions. Remarks are included upon the KIO_3 method of Busch.**

Among histologic methods employed upon the central neural system, the method of Marchi has remained peculiarly refractory to improvement. Altho some fundamental information has been acquired by Swank and Davenport[1] in regard to cat and rabbit material, and certain modifications of the method have been suggested for particular purposes, the essential difficulty of the technic, notably poor penetration, has not been circumvented. In so far as brain material itself is concerned, especially primate specimens, we still find the method described ten years ago in this journal[2] to be the most useful for general laboratory purposes, but many critical sections have had to be discarded because of poor penetration.

Whereas it is generally admitted that good penetration can usually be obtained upon slices of tissue 3 mm. or less, it is not always advisable to cut large brains into such thin slices because of subsequent warping while in the osmium solution. In an effort to increase the penetration of the osmium-dichromate mixture we have consequently conducted a series of experiments with surface tension depressants. Of these the most useful is "Tergitol 08" (Carbide and Carbon Chemicals Corporation, 30 East 42nd Street, New York,

[1]Swank, R. L., and Davenport, H. A. 1934–5. Marchi's staining method: I. Studies of some of the underlying mechanisms involved; II. Fixation; III. Artefacts and effects of perfusion. Stain Techn., **9**, 11–9, 129–35; **10**, 45–52.

[2]Mettler, Fred A. 1932. The Marchi method for demonstrating degenerated fiber connections within the central nervous system. Stain Techn., **7**, 95–106.

N. Y.). This may be added directly to the 3% dichromate solution which is used for both the preliminary treatment and in making up the osmium mixture. It should be used in the proportion of one part of "Tergitol" to 150 parts of 3% dichromate. While the effect of a surface tension depressant seems to be beneficial, the improvement in penetration is not great enough to warrant recommending it as a modification of the routine procedure.

Among the modifications suggested by Swank and Davenport we have had the best penetration by the use of the following preliminary procedure. The animals (monkeys) were killed by an overdose of "Nembutal" and were perfused with a mixture of Mueller's fluid and $CuSO_4$. The brains were fixed in formalin. Altho a slightly better degree of penetration was encountered after such a procedure than after the routine technic, the difference was not marked and the tissue had a tendency to overstain. In cases in which we perfused the brain with $MgSO_4$ after death from "Nembutal" and then fixed in formalin followed by the chlorate mixture, the infiltration was very poor and the staining of the penetrated areas was suppressed. Brains from monkeys killed by exsanguination and fixed in formalin, followed by staining in a mixture of $KClO_3$, acetic acid, formalin and OsO_4 exhibited a tendency toward overstaining and underpenetration.

Swank has pointed out that good results can be obtained by transferring unperfused brains, fixed in formalin, directly to the Marchi mixture (equal parts of 3% $K_2Cr_2O_7$ and 1% OsO_4) without previous washing. Such a technic is applicable to primate material and is especially useful on monkeys that have not run the full degeneration period of two weeks. Altho we regard washing after formalin fixation and treatment with plain $K_2Cr_2O_7$ as an optional procedure, the results obtained are usually more uniform if these steps are included. Washing out of the formalin certainly reduces the tendency toward surface blackening (the brain should never be allowed to fix in formalin alone for more than 24 hours) and a stay of two weeks in $K_2Cr_2O_7$ improves the quality of the background of the finished sections. The use of plain $K_2Cr_2O_7$ can, however, be a source of trouble in that if the tissue is left in this solution too long the staining reaction is suppressed. Further, if the $K_2Cr_2O_7$ is too concentrated, the blocks of tissue become hard and recalcitrant to embedding. Very thoro washing after treatment with the osmium mixture is a necessity if good pyroxylin infiltration is to be attained.

Testing the strength of the Marchi mixture. The tendency to conserve a reagent as expensive as osmium is no doubt responsible for many cases of insufficient staining. One should always use at least

twenty times as much of the osmium-dichromate mixture as the volume of tissue to be stained, and results are likely to be more satisfactory if fifty times the tissue volume is employed. When one is working with primate brains and the cost of osmium involved is considerable, one is loathe to dispose of the partially exhausted fluid after the brain is removed from it. So far as we are aware, no one has ever suggested a method by which the unused osmium may be reclaimed. Some workers have long used weakened mixtures in place of straight 3% $K_2Cr_2O_7$ in the preliminary treatment, but this does not represent any saving in the cost of the total technic.

Not only would it be desirable to know how much osmium remains in such a mixture, but it must be admitted that there is no satisfactory way of knowing if the osmium-dichromate mixture becomes exhausted during the two-week period while the tissue is supposed to be staining. Few people can tell much about the concentration of the Marchi mixture by smelling it. A method for the detection of osmium has been described by Pinerua Alvarez[3] and can be modified to serve this purpose. The modified procedure is as follows: To 1 cc. of the osmium-dichromate mixture of unknown strength add 250 mg. of barium acetate powder, centrifuge for 10 minutes and separate the supernatant fluid from the precipitate. In order to wash out the OsO_4 which may have been adsorbed by the resulting $BaCrO_4$ precipitate, add 1 cc. of acetic barium acetate solution (saturated solution of barium acetate diluted with water 1:5 and further acidulated by the addition of a few drops of glacial acetic acid to each 100 cc. of the solution) to the precipitate, mix thoroly and centrifuge for 10 minutes. Add the supernatant fluid to that previously obtained and add 4 drops of the test solution (2 parts by volume of 20% KI plus one part of concentrated HCl). A deep emerald green color indicates the presence of osmium. While the test solution can be added directly to a sample of the Marchi mixture, it is preferable to remove the $K_2Cr_2O_7$ as indicated above[4] since it obscures the color developed in the actual test. The intensity of this color is roughly proportional to the amount of osmium present and, in laboratory practice, it will be found desirable to run three samples, notably the osmium-dichromate of unknown strength and two samples of known strength (one containing equal parts of 3% $K_2Cr_2O_7$ and 1% aqueous OsO_4, and the other containing equal parts of 3% $K_2Cr_2O_7$ and 0.5% OsO_4). In this way the unknown can be compared against standards representing 0.5% and 0.25% of OsO_4

[3]Alvarez, P. 1905. Sur un nouveau composé osmieux et une réaction de l'osmium. Compt. Rend. Acad. Sci., **140**, 1254–6.

[4]We are indebted to Dr. H. B. Waelsch for assistance in this matter.

respectively. For effective staining the osmium concentration should not be allowed to drop below 0.25%.

The standards should be run at the same time as the unknown since the color of the reaction is unstable and comparison would otherwise be impossible. It is necessary to use standards containing $K_2Cr_2O_7$ since a certain amount of osmium is lost during the extraction of this substance from the unknown. If the standards also contain $K_2Cr_2O_7$ and are subjected to the extraction process, the loss is identical in each case and may be ignored in the final comparison.

Reclaiming partially exhausted staining mixtures. When one has finished staining in a particular osmium mixture, the residual osmium may be conserved in the following manner: Centrifuge to eliminate debris and compare colorimetrically the partially exhausted Marchi mixture with a 1.5% solution of $K_2Cr_2O_7$. If the percentage of $K_2Cr_2O_7$ has previously been decreased by the addition of significant amounts of 1% OsO_4, it will now be necessary to adjust this by the addition of sufficient 3% aqueous $K_2Cr_2O_7$. Test the osmium concentration as previously indicated. If the concentration of osmium is below 0.25%, it will probably not be worth while to attempt to adjust the solution to standard strength unless the volume is very large. The strength of osmium may now be brought up to the required amount by the addition of solid OsO_4 or a solution of this. If much 1% solution is added, the $K_2Cr_2O_7$ concentration has to be adjusted; so, in practice, it is better to work with amounts of partially exhausted solution aggregating a liter or more. In this case the amount of solid necessary to reënforce the solution can be computed without much difficulty, and it is consequently unnecessary to disturb the dichromate concentration. If frequently reused, the $K_2Cr_2O_7$ of any given Marchi solution will become reduced and ineffective. This will be quickly noticed since the solution appears greenish. Altho a slightly green mixture is still active and no harm is done if the change occurs while the tissue is being stained, such a solution should not be used again.

Embedding of Marchi material. Since the conventional paraffin solvents have a generally deleterious effect upon Marchi material, the pyroxylin technic is usually employed. Impregnation with a celloidin-like substance is frequently difficult to carry out on dichromate material. It is probably always advisable to remove pia by slicing away the periphery of a block wherever possible. If the block is large and this is impossible, it is better to use the method of Busch[5]

[5]Busch, C. K. 1898. Über eine Färbungsmethode secundärer Degenerationen des Nervensystems mit Osmiumsäure. Neurol. Zentbl., **17**, 476.

since iodate material is less resistant to infiltration than dichromate material. If one insists upon the dichromate technic, good sections can always be obtained by taking one's time with the process of embedding. We have left Marchi material in 2% celloidin as long as a year and still obtained good sections. It is unnecessary to be in a hurry during embedding and impatience may be disastrous.

In spite of theoretic objection, celloidin blocks of Marchi stained material may be stored in glycerin and alcohol for a short time without much impairment in the contrast of the stain. We do not recommend leaving them more than a month. If it is desired to keep the tissue in blocks longer than this, terpineol is preferable. Terpineol or butyl alcohol may be used after 90% alcohol to clear sections which are difficult to carry thru absolute alcohol. All clearing agents, in time, affect the stain to some extent. In addition to those mentioned as useful in our 1932 article, we may now add oil of origanum which is better than carbol-xylene but not so good as $CHCl_3$. If one employs a quick drying mount, such as damar instead of balsam, the solvent action of the clearing agent will be found to be noticeably reduced.

Bleaching. The attempt to bleach overstained sections is generally a useless procedure. The photographic reducers, whether proportional or cutting, do not have any appreciable effect upon such sections, but H_2SO_3 or K_2SO_3 may be tried if one happens to have a few critical sections which it is desirable to salvage.

H_2O_2 is of no value in bleaching Marchi sections, but is a very useful reagent to have on hand. Occasionally a stock bottle of 1% aqueous solution of OsO_4 will become darkened. Under ordinary circumstances such a solution would be discarded, but this is unnecessary since the addition of a small amount of H_2O_2 not infrequently restores the water-like clarity of the solution. Of course, organic material and metals should be kept out of stock bottles.

Degeneration. Occasionally, when a suitable animal dies before the appointed time set for degeneration, one is tempted to stain the material in the hope that a usable result will be obtained. This practice is to be deprecated as a waste of time and osmium. What one gets, under such circumstances, is a little local staining about the immediate lesion and nothing more. Cut myelinated fibers seem to become osmium-susceptible rather suddenly. In mammals this does not appear to occur earlier than the tenth day. After three weeks good results are secured with primate material and acceptable sections can be obtained as long as five weeks after lesion. (We do not consider the possibility of retrograde degeneration to be a serious

enough objection to discard material which has run for this length of time; tho the record of the case should always clearly state the time period involved). If one is forced to stain before the lapse of the optimal period, Swank's recommendation of eliminating preparatory mordanting in 3% $K_2Cr_2O_7$ should be adopted, but negative results should not be reported on such material.

Potassium iodate technic (method of Busch). The use of KIO_3, in place of $K_2Cr_2O_7$, possesses the advantage of more complete pyroxylin infiltration and consequently greater speed. It has the disadvantage of giving an exceptionally pale background (which may, however, be counterstained).

We have found the following to be the best procedure: (1) fix in an aqueous solution of 10% formalin and 1.5% KIO_3 for about 24 hours; (2) rinse briefly, cut into slices not above 4 mm. thick and place directly in an aqueous solution consisting of 2 parts of 1.5% KIO_3[6] and one part of 1% aqueous OsO_4 solution; (3) after infiltration with the osmium-iodate mixture for about two weeks, wash thoroly in running water until all traces of the KIO_3 have been removed, dehydrate in short steps, slowly and completely, and run up in pyroxylin, remembering that most of the actual infiltration occurs in the weakest concentrations. Counterstain, if desired, with carbolfuchsin or safranin.

When the method has been properly applied, the degree of background protection obtained by the iodate technic exceeds that resulting from the use of dichromate. If the KIO_3 has decomposed, the osmium will stain the interior of the block indiscriminately and only the periphery will show adequate protection.

[6]Caution: do not use $KH(IO_3)_2$ and do not mix the KIO_3 until ready to use since it deteriorates and proportional protection of normal tissue will not be obtained. Further, do not wash between steps 1 and 2 since this is likely to cause considerable softening and consequent distortion. A preliminary iodate solution placed between simple formalin fixation and the Busch mixture should not be used but, if the iodate was omitted from the fixer, go, after a single water rinse, directly to the Busch mixture from formalin.

A FLAGELLA STAINING TECHNIC FOR SOIL BACTERIA

PAUL J. FISHER AND JEAN E. CONN, *Department of Bacteriology, New York Agricultural Experiment Station, Geneva, New York*[1]

ABSTRACT.—**In an attempt to stain the flagella of soil bacteria, many of which have flagella so fine that they are hard to stain by most methods, a technic was developed which combines the best points of Hofer and Wilson's method with that of Bailey as developed by O'Toole. Satisfactory preparations have been obtained for organisms of the genera *Pseudomonas*, *Phytomonas*, *Alcaligenes*, *Escherichia*, *Azotobacter*, and *Bacillus*. This technic, therefore, is recommended as a rapid and constant method for routine flagella staining of all motile aerobic organisms; it combines Hofer and Wilson's method of cleaning the slides with O'Toole's technic of spreading the smears and with a modification of Bailey's mordant.**

This technic was developed in an attempt to stain soil bacteria, many of which have flagella so fine that they are hard to stain by the methods previously described. Altho much has been written about flagella staining, very little work has been done with soil bacteria. Many of the common soil and water bacteria have been described as motile but with no flagella reported. With other organisms, flagella have been observed, but the type of flagellation has been reported differently by various investigators, probably because the cells and the flagella are so small that the staining is not satisfactory. These difficulties indicated that further work along this line was necessary. One step in this direction was the modification of Gray's method (1926) by Hofer and Wilson (1938) for staining slime-forming organisms. This was further modified by Conn and Wolfe (1938) for use with other types of bacteria. This technic has several disadvantages. First, the method employed in spreading the smears results in so few bacteria per field that a great deal of time is required for the examination of one slide. Furthermore, the method has proved to be extremely variable, possibly due to the use of saturated solutions in the mordant. In addition, the background is often heavily covered with precipitate.

In searching for a better method, that of Bailey (1929), as modified by O'Toole (1942) for use with anaerobes, was tried. Altho

[1]Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 512.

this technic had several advantages over the others, it was not found to be suitable for bacteria having fine flagella.

In the present study, the test organism was an unidentified pseudomonad which was actively motile in hanging drop but (probably because of unusually fine flagella) was difficult to stain by all methods tried. In an attempt to stain this organism, the above methods were modified and combined in various ways. Different combinations of the chemicals were tried in the mordant. Those which proved most satisfactory were tried in different concentrations, and solutions of a definite strength were substituted for the saturated solutions (which vary with temperature changes). Finally, the most satisfactory length of time for each step was determined.

Recommended Procedure

As a result of this work the following method is recommended.

Cleaning of slides: Use new slides if available. Wash in dichromate cleaning solution; rinse in distilled water; transfer to 95% alcohol. Upon removal from the alcohol, heat the slide by passing back and forth thru a flame until a yellow color appears in the flame. Gradually lift the slide above the hottest part of the flame and then place, flamed side up, on a metal plate, heated just hot enough to produce steam from water. Allow the plate to cool gradually in order to re-anneal the slide and thus prevent breakage. Some experience is required to judge the amount of heating necessary. This part of the technic is a modification of that described by Hofer and Wilson.

Preparation of cultures: Use young actively growing cultures on agar slants (the exact age depends upon the species studied; approximately 18 hours' incubation is satisfactory for most organisms). Before proceeding, check the culture for motility by the hanging drop method. Wash off the growth by gentle agitation with 2–3 ml. of sterile distilled water. Transfer the suspension to a sterile test tube and incubate at optimum temperature for 10 minutes (30 minutes for those producing slime). At this point, again observe the suspension for motility. Transfer a small drop of the culture from the top of the suspension by means of a Pasteur pipette to one end of the previously cleaned and cooled slide. Gently tilt the slide and allow the drop to run slowly to the other end. When properly spread, there is room for three such streaks on a slide. Place the slide in a tilted position and allow to dry in the air. This method of spreading was first described by O'Toole, and gives better results than the method of Conn and Wolfe. The reason for this is that O'Toole's technic gives a longer margin per unit area than the other method,

and since it is a well-known fact that motile organisms tend to migrate toward the edge of the smear, it is comparatively easy to find a good field.

Mordanting: The method used in mordanting is a modification of the Bailey-O'Toole procedure, which requires two solutions that should be prepared fresh each time.

Solution I

Tannic acid, 10% aqueous	18 ml.
Ferric chloride, 6% aqueous	6 ml.

Solution II

Solution I	3.5 ml.
Basic fuchsin, 5% alcoholic	0.5 ml.
HCl, concentrated	0.5 ml.
Formalin	2.0 ml.

In this formula, the most important modification is the substitution of a 5% alcoholic solution of basic fuchsin for Ziehl-Neelsen's carbol fuchsin as recommended by Bailey. The mordant is applied as follows: Pour Solution I onto the slide thru a filter and allow it to remain for 3.5 minutes. Pour off this solution, and without washing, add Solution II in the same way. After 7 minutes, wash the slide with distilled water. These solutions may be refiltered and used again as many times as desired in any one day, but must not be kept overnight.

Staining: Before the slide dries, cover with Ziehl-Neelsen's carbol fuchsin, and allow it to stand for one minute on a hot plate heated just enough for steam to be barely given off. (This is essentially as recommended by Bailey). Wash and dry the slide in the air.

The time required for the first step of the mordant and for the staining should be carefully controlled because a slight variation either way may have a marked effect on the amount of precipitate and the intensity of staining.

Organisms used: In addition to the pseudomonad used as the test organism, this method was tried with *Alcaligenes fecalis*, *Phytomonas tumefaciens*, *Phytomonas savastanoi*, *Pseudomonas fluorescens*, *Escherichia coli*, *Azotobacter vinelandii*, and *Bacillus subtilis*[2]. The above organisms were chosen as representatives of the major groups of motile aerobic bacteria.

[2]The organisms listed were obtained from the following sources: *Alcaligenes fecalis*, Dr. Carl Nyberg, Helsinki, Finland; *Phytomonas tumefaciens*, Dr. Stapp, Berlin, Germany; *Phytomonas savastanoi*, Clayton Smith, Riverside, Calif.; *Pseudomonas fluorescens*, stock culture, N. Y. Agricultural Experiment Station; *Escherichia coli*, American Type Culture Collection; *Azotobacter vinelandii*, N. R. Smith, Washington, D. C.; *Bacillus subtilis*, E. H. Wheeler, Hobart College, Geneva, N. Y.

Results

The test organism, when stained by this method, proved to have a single, fine, very long, polar flagellum; the authors, therefore, believe that it belongs to the genus *Pseudomonas*. Photomicrographs of this organism are shown in Fig. 1. *Pseudomonas fluorescens*

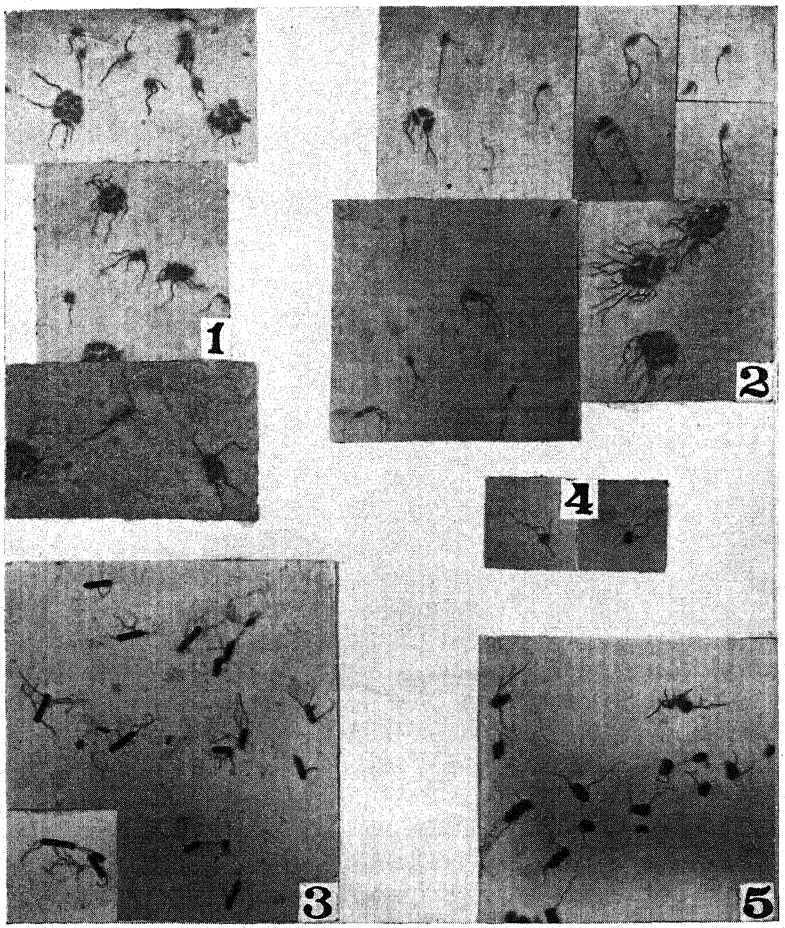


Fig. 1. The test organism, an unidentified species of *Pseudomonas*. ×900.
Fig. 2. *Pseudomonas fluorescens*. ×900.
Fig. 3. *Alcaligenes fecalis*. ×900.
Fig. 4. *Escherichia coli*. ×900.
Fig. 5. *Azotobacter vinelandii*. ×900.

was easier to stain, and many fine preparations were obtained (Fig. 2). Excellent results were obtained with *Alcaligenes fecalis*,

which proved to be a good example of an organism having "degenerate peritrichous" flagellation (Fig. 3). The flagella on the two species of *Phytomonas* showed up very nicely, altho both the organisms and the flagella were so small that no good pictures of them were secured. This method, like most other flagella staining technics, worked well with *Escherichia coli* (Fig. 4). *Azotobacter vinelandii* showed large cells with "degenerate peritrichous" flagellation which was fairly easy to demonstrate by staining (Fig. 5). This organism has previously been described by Lipman (1903) as having a single polar flagellum. The staining of *Bacillus subtilis* was not difficult; the only problem encountered was that of obtaining the culture at the right age.

Conclusions

This staining technic proved to give consistently good results, and showed the flagella distinctly outlined against a clear background. Even those species of *Phytomonas* and *Pseudomonas* that have flagella which are not readily observed by other methods are easily stained by this technic. Since satisfactory preparations were obtained for such a wide variety of organisms, it is reasonable to assume that the method can be used for any motile aerobic species.

REFERENCES

Bailey, H. D. 1929. A flagella and capsule stain for bacteria. Soc. Exp. Biol. and Med., **27**, 111–2.

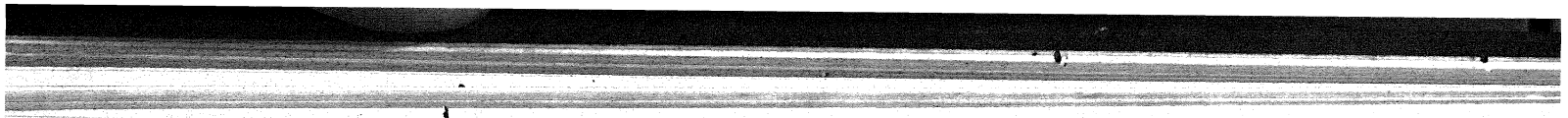
Conn, H. J., and Wolfe, Gladys E. 1938. Flagella staining as a routine test for bacteria. J. Bact., **36**, 517–20.

Gray, P. H. H. 1926. A method of staining bacterial flagella. J. Bact., **12**, 273–4.

Hofer, A. W., and Wilson, J. K. 1938. Use of the Gray flagella stain for slime-forming bacteria. Stain Techn., **13**, 75–6.

Lipman, J. G. 1903. Transformation and fixation of nitrogen by bacteria. N. J. Agr. Exper. Station Report, **24**, 217.

O'Toole, Elizabeth. 1942. Flagella staining of anaerobic bacilli. Stain Techn., **17**, 33–40.



A RAPID SILVER-ON-THE-SLIDE METHOD FOR NERVOUS TISSUE[1]

MAURICE L. SILVER, *Hull Laboratory of Anatomy, University of Chicago, Illinois*

ABSTRACT.—A progressive silver staining method is described, which permits microscopic examination of the sections during the staining process. After formaldehyde fixation, dehydration and embedding in paraffin or celloidin, fine fibers and synaptic endings may be demonstrated. After formaldehyde fixation and mordanting in 3% $K_2Cr_2O_7$, myelinated fibers and mitochondria are specifically stained.

The unique feature of this method is, that the silver solution (0.5% protargol) is mixed with the reducing solution: 1.6% Rochelle salts, containing traces of Ag NO_3, $MgSO_4$, and K_2S (U.S.P.). The sections are placed directly into this mixture, which is then warmed to 45–55° C. Sections are removed when progressive staining is completed, washed in water, dehydrated and mounted.

In the fiber stain, nerve fibers and synaptic endings are dark brown or black, and nuclear chromatin is deep brown, against a pale yellow background. When the myelin sheath procedure is followed, the fiber bundles are deep brown, and the intensity of the staining remains the same for specific tracts, aiding in their identification.

This method offers the advantages of speed, simplicity, and progressive staining in the demonstration of fine fibers and synaptic endings in the nervous system.

With appropriate modification in fixation and dehydration, it may also be used as a specific stain for myelinated fibers. The theoretical considerations underlying the use of this, and other silver methods, have already been discussed. (Silver, 1942).

The silver solution used in this method, protargol (silver albumose), is not new in histological technic. It was first used by Regaud and Dubreuil (1903), rediscovered by Bartelmez and Hoerr (1933), and since used by Bodian (1936), Davenport *et al.* (1938, 1939), and others.

The reducing solution used in this method is new to histological technic. It is derived from an old formula for silvering glass, for

[1]This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

which I am indebted to my friend, Victor Shanok, who initiated me into the fine art of chemical reduction of silver. It differs from other reducing solutions used in histological silver methods in composition, and especially in its method of use. It is mixed directly with the silver solution, the slides are immersed in the warmed mixture, staining is progressive, and in 2–3 hours the stain is completed.

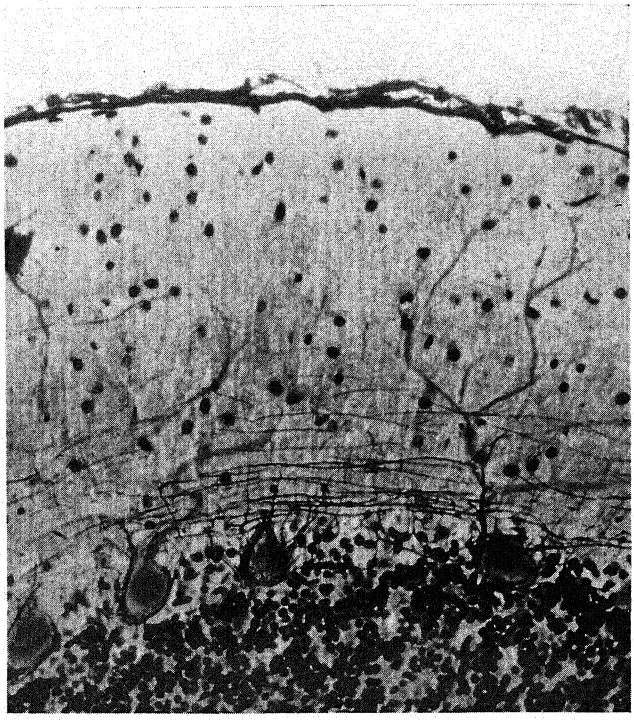


Fig. 1. Photomicrograph of a 12 μ, celloidin section of the cerebellar cortex of a monkey, stained with the silver method described in the text for fine fibers. This tissue was fixed in a formalin-alcohol-acetic-acid mixture (Formol: 10%; Acetic acid: 2%; in 80% ethyl alcohol), dehydrated in higher alcohols for a month, imbedded in celloidin, cut, and mounted on the slide with the clove oil technic. Celloidin was removed with two changes of ether-alcohol and acetone, the slide placed in water, and stained 3 hours with the protargol-Rochelle-salts method at a temperature of 52°C. ×200.

Note the basket fiber endings around the perikaryon of the Purkinje cells.

The method may be used to stain nuclei, fine fibers, and neuronal terminations as follows:

1. Fix tissues, preferably by perfusion with 10% neutral formalin in 1% NaCl, 10% commercial formalin in 1% NaCl, formalin-formic-acid, formalin-alcohol, formalin-acetic-alcohol, Bouin's fluid, etc.

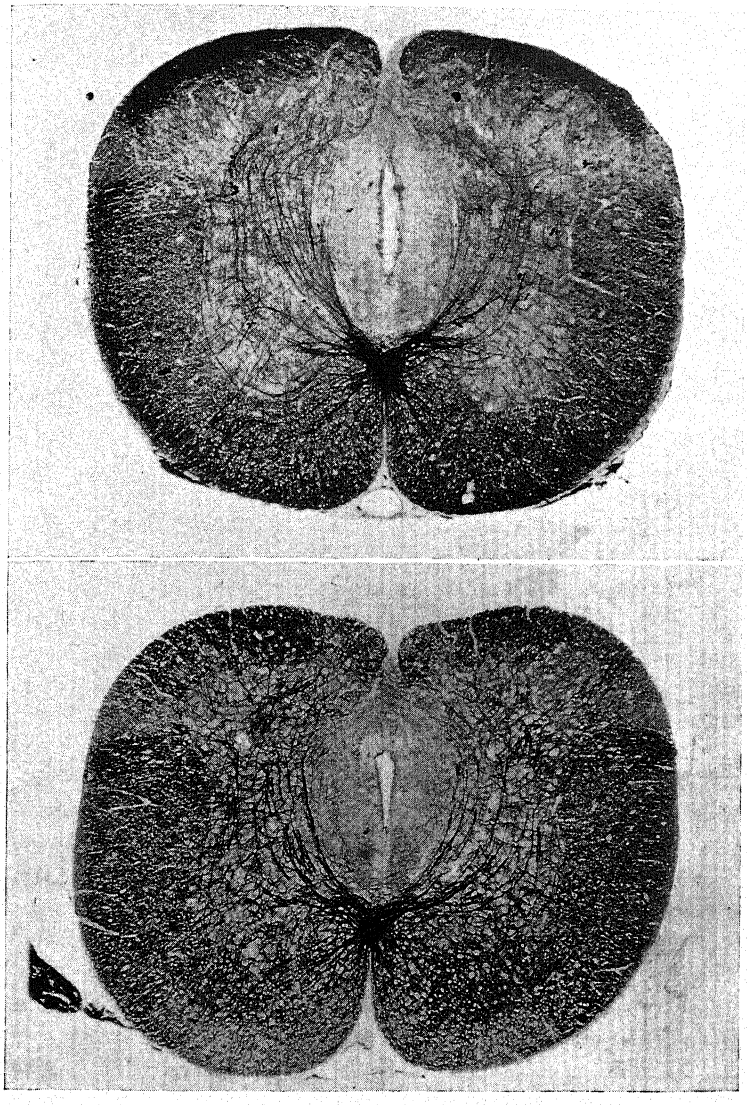


Fig. 2. Photomicrographs of two 20 μ, paraffin-imbedded, transverse sections of the medulla oblongata of the frog at the level of the most caudal end of the IV ventricle. This block was fixed in Regaud's formol-bichromate mixture, and mordanted for a week in 3% $K_2Cr_2O_7$. The tissue was washed in water for 24 hours, dehydrated in increasing concentrations of ethyl alcohol, double-imbedded in 3% celloidin-paraffin, and cut at 20 μ. $\times 40$.

The upper section was stained on the slide with the Weigert method and the lower section was stained on the slide with the silver method described in this paper for myelin sheaths.

Note that the silver-stained section shows more of the finely myelinated fibers than the upper Weigert specimen, and also differentiates the large fiber tracts in the white matter.

2. Wash, dehydrate slowly, imbed, cut sections at 2–20 μ, and mount on the slide; or frozen sections may be cut, 10–40 μ.

3. If imbedded in paraffin, remove paraffin with xylol and run sections to water. If imbedded in celloidin, remove celloidin with changes of acetone and absolute-alcohol-ether, run sections to water.

4. Immerse slides or sections in mixture of equal parts of (a) 0.5% aqueous protargol[2] and (b) Rochelle salts reducing solution, prepared as follows:

Dissolve 64 g. Rochelle salts (potassium sodium tartrate) in 500 cc. distilled water. Boil vigorously. Add 10 cc. of 10% $AgNO_3$. Boil with stirring for at least 5 minutes. Remove the flame. Add 0.3 g. crystalline $MgSO_4$; then while simmering add 0.2 g. K_2S, (U.S.P.) using only the brown, unoxidized portion of one piece. Filter while hot, and add distilled water to make a total volume of 4 liters.

Rochelle salts reducing solution thus prepared will make 4 liters of reducing agent, and improves slightly with age. (We have used one stock for more than a year with absolutely constant results on similarly fixed tissue).

5. The protargol-Rochelle-salts mixture containing the sections is placed in the incubator or on a hot plate, at a temperature of 45–55°C. Staining is progressive, and usually completed in 2–3 hours. Slides may be removed during the process and examined, a feature which makes this procedure relatively unique among silver methods.

6. When staining is completed, usually before there is grossly visible reduction of the silver in solution, the slides are removed, washed in two changes of distilled water, dehydrated and mounted.

Figure 1 is a photomicrograph of monkey cerebellum stained with the above method to demonstrate fine fibers.

The method may be used to stain myelin sheaths and mitochondria, as follows:

1. Fix tissues, preferably by perfusion, with 10% formalin in 1% $K_2Cr_2O_7$, or with 10% formalin in 1% NaCl.

2. Mordant small blocks of tissue in 3% $K_2Cr_2O_7$ for one week. This step may be omitted if the tissue has been in the fixative for more than a week.

3. Wash, dehydrate, imbed, cut sections at 4 to 20 μ, and mount on the slide.

4. Remove imbedding medium, and proceed as above.

Figure 2 is a photomicrograph of the medulla oblongata of the frog, stained to demonstrate myelinated fibers. The upper section

[2]Protargol, strong protein silver (silver albumose), manufactured by the Winthrop Chemical Company Inc., New York, N. Y.

was stained by the Weigert method, and the lower section was stained with the silver method described in this paper for myelin sheaths. Note that it shows even more of the finely myelinated fibers than are demonstrated by the standard Weigert method on the same material.

The staining of the myelin sheaths with this method is specific in intensity for specific fiber-tracts, and aids in their identification when they are followed in successive sections.

REFERENCES

BARTELMEZ, G. W., and HOERR, N. L. 1933. Vestibular club endings in *Ameiurus;* further evidence on the morphology of the synapse. J. Comp. Neurol., **57**, 401–28.

BODIAN, D. 1936. New method for staining nerve fibers and nerve endings in mounted paraffin sections. Anat. Rec., **65**, 89–97.

DAVENPORT, H. A., and KLINE, C. L. 1938. Staining paraffin sections with protargol; experiments with Bodian's method; use of *n*-propyl and *n*-butyl alcohol in Hofker's fixative. Stain Techn., **13**, 147–60.

DAVENPORT, H. A., MCARTHUR, J., and BRUESCH, S. P. 1939. Staining paraffin sections with protargol: optimum pH for reduction; 2-hour staining method. Stain Techn., **14**, 21–6.

REGAUD, CL., and DUBREUIL, M. 1903. Sur un nouveau procédé d'argentation des epitheliums au moyen du protargol. Compt. Rend. Assoc. Anat., 5th session, Suppl., 121–3.

SILVER, M. L. 1942. Colloidal factors controlling silver staining. Anat. Rec., **82**, *Suppl.* 507–29.

A STAINING RACK FOR HANDLING COVER-GLASS PREPARATIONS

TZE-TUAN CHEN, *Department of Zoology, University of California at Los Angeles*

A porcelain staining rack (Fig. 1) has been devised for handling

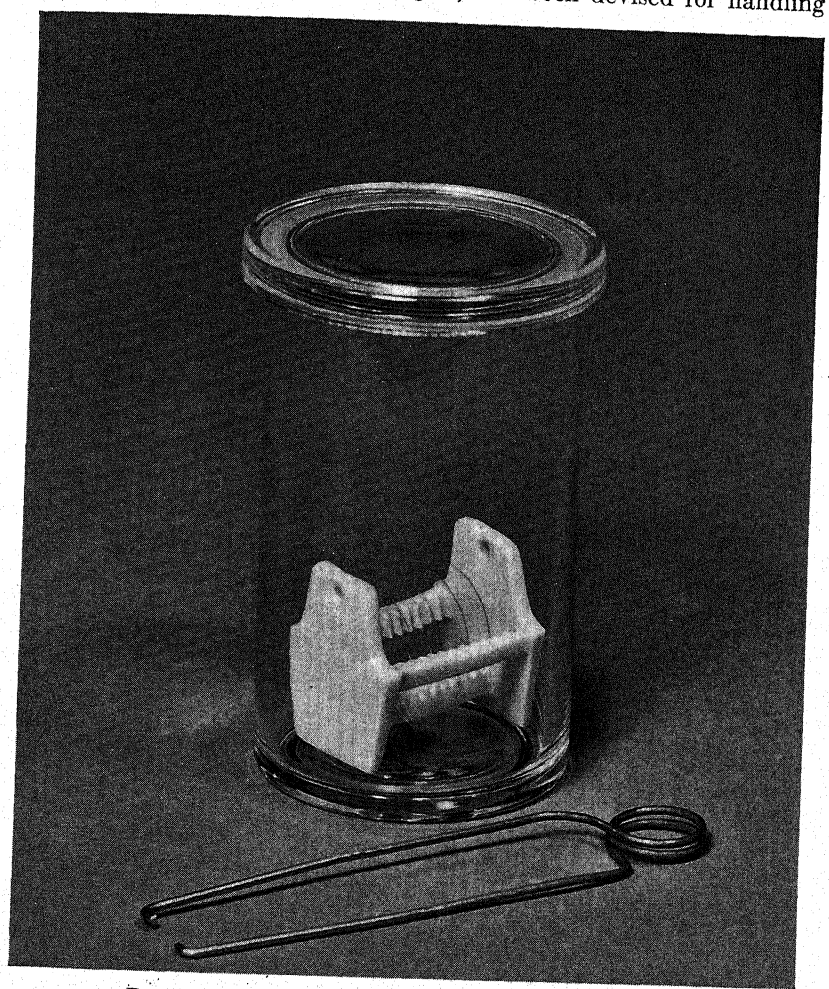


FIG. 1. Photograph of the staining rack in a Stender dish. Wire tongs or handle in front of the dish.

cover-glass preparations. The design is along the general lines of staining racks for slides commonly sold by commercial houses. It

consists of three parallel rods—one at the bottom, two on the sides. These three rods are held together by two end pieces. Each of the rods has twelve slots, thus the rack holds twelve cover glasses. The slots have been arranged to hold the cover glasses some distance apart. Circular cover glasses having a diameter of 22 mm. are most desirable. Each of the two end pieces has a hole near the top for inserting one end of the wire tongs or handle (see Fig. 1) which is used for removing the rack from the stender dish. The staining rack is 40 mm. long and 35 mm. high and is designed, as shown in the figure, to fit the ordinary Stender dishes commonly used in many laboratories.

The obvious advantages of this rack in staining, destaining, and dehydration of cover-glass preparations are: (1) Twelve cover glasses can be handled at once, thereby saving much time. (Larger racks to handle more than twelve cover glasses at a time can be easily designed.) (2) There should be less danger of breakage of cover glasses and of damage to the specimens (especially those near the edges of cover glasses). (3) The staining rack seems more convenient than the so-called "Columbia cover-glass staining dish" designed by Bowen.[1] It is believed that many protozoölogists, parasitologists, some cytologists, and perhaps others should find the staining rack particularly useful.

This staining rack for cover glasses can now be purchased from Arthur H. Thomas Company, West Washington Square, Philadelphia, Pennsylvania, whose courtesy and cooperation is hereby acknowledged.

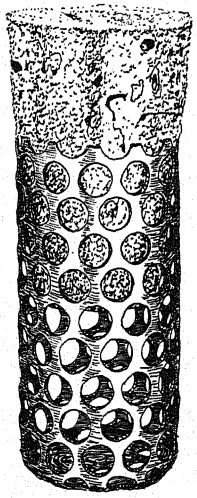
[1]Bowen, R. H. 1929. A new staining dish for handling cover-glass preparations. Stain Techn., 4, 57–8.

NOTES ON TECHNIC

The Washing Bobber

The small gadget now described has been in use for some years in this laboratory for washing histological specimens. Its extreme simplicity and cheapness is of the greatest advantage when we have to cope with a large number of preparations for class work. It serves its purpose admirably and is just as effective as many other elaborate pieces of apparatus described elsewhere.

The bobber illustrated in Fig. 1 is made as follows. A rectangular piece of perforated zinc is bent round a cylindrical piece of metal or wood of the required diameter. The ends, which may overlap or butt against one another, are soldered. The open tube is based by soldering a small piece of the same metal to it, the rough edges are then trimmed off with shears and the bobber is complete. We have found the most convenient size to be $1\frac{1}{2}''$ high by $\frac{3}{4}''$ diameter. The tissue is put in the device with a small pencilled label if necessary and the open end is closed with a cork. It is then thrown into a bowl in a sink under a stream of running water from the tap. The bobber moves along the surface and then comes under the jet of water when it suddenly dives and reappears at the periphery of the bowl; this circular motion is repeated over and over again. It was this rapid down and up movement that led our students to christen it "the bobber"; it ensures very thoro washing of the tissue. If necessary, up to twenty bobbers can be accommodated in an ordinary sized bowl without lessening the effects of the washing.—F. W. Gairns, *Institute of Physiology, University of Glasgow, Scotland.*



Clarite in Embedding Paraffin for Thin Sections

During the course of preparing slides of *Triturus* larvae of 15 mm. and less, the writer had to work in a room too warm for sections in ordinary paraffin mixtures. As the embedding oven used was employed by a number of other people, it was not convenient to embed in very high melting-point paraffin. It was discovered that addition

of small amounts of clarite (Nevillite V) provided a much harder block and by varying the amounts added, sections down to 2–3 μ might be cut at ordinary room temperature. With a mixture of 90% paraffin 53°C. m.p., 5% bleached beeswax, and 5% clarite, by weight, satisfactory sections may be made in a warm room. With this mixture, however, the ribbons normally become charged with electricity more than ordinary ribbons so that a spark-coil device such as described by Blandau[1] should be used.—WILLIAM WEHRLE[2], *Berkeley, Calif.*

[1]Blandau, R. J. 1938. A method of eliminating the electrification of paraffin ribbons. Stain Techn., **13**,139–141.

[2]Formerly employed by the U. S. Works Progress Administration. Aid from this Administration (Official Project 65–108–113, Unit C-1) is hereby acknowledged.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

POLYAK, S. L. **The Retina.** 7½ x 10½ in. 608 pp. + 114 pp. of plates. Cloth. University of Chicago Press. 1941. $10.00.

The principal object of this elaborate book is the elucidation of the complex structure of the retina and the interpretation of the basic visual functions in terms of structures of the visual organs. The book is divided into five parts and thirty-four chapters. Part I deals with the histological technic used by the author. The Golgi-Cox method (with $HgCl_2$ instead of $AgNO_3$) was used because of greater uniformity of impregnation. The other procedure used was the methylene blue stain of Ehrlich by injection of the dye into the vitreous of living animals. The disturbing factor of both methods is their irregularity. The methods do not stain all the elements of a tissue simultaneously but rather in a haphazard way, picking up here and there one or another of its particular constituents. Conclusions concerning morphological interrelationships as well as functional mechanisms are not always drawn with extreme care, as should be done because of the uncertainty of methods used. Parts II and III deal with a survey of investigations from ancient to contemporary times. The most valuable part of this work seems to be the minute description of the various nerve cell elements of the retina with their synapses (part IV). Part V deals with the functional interpretation of retinal structures. The neuroglia is considered an insulator of neurons and their expansions. Small asteriform elements confined to the territory of the seventh layer are recognized as microglia. The theory of a continuous glial syncytium is called "a phantasmagoria fathered by prejudice and mothered by lack of experience". The author is strongly opposed to the doctrine of the nerve nets (neurencytium) and adheres to a somewhat modified neuron doctrine. A number of illustrative photomicrographs and drawings present a very valuable addition to the text.—*Gabriel Steiner.*

MICROSCOPE AND OTHER APPARATUS

COOK, F. W., SATTERFIELD, G. H., and RALEIGH, M. A. **Multiple tissue washer and processing assembly. Combination apparatus facilitating the preparation of tissues for paraffin section.** *J. Lab. & Clin. Med.*, 27, 673–9. 1942.

The tissue washer consists of a deep, 20-gauge, 6-qt. aluminum pudding pan perforated with 27 holes joined to short copper tubes with solder, each holding a 7-in. length of rubber tubing provided with a Hoffman clamp. Thus a reservoir of water from a single tap is provided for from 1 to 27 assemblies. The washer is durable, inexpensive, easily constructed, requires a small space and gives a regulated flow for each tissue container.—*John T. Myers.*

CRABB, EDWARD D. **A multicolored wax light filter and dissecting chamber holder for microscopes.** *Rev. Sci. Instruments*, 12, 154. 1941.

The base for this combination light filter and watch glass holder is a synthetic wax, known commercially as "Flexowax C," which is colored with oil-soluble dyes. The wax and six "Glycolors" (black, blue, brown, green, red and yellow) may be obtained from Glyco Products Company, 148 Lafayette Street, New York.

In combining the colors a small quantity of Flexowax C was melted and the dye added until a deep color was obtained. After cooling, the plastic wax was rolled into small balls and stored until needed in separate shell vials. Additional colors

were obtained by kneading small pieces of two or more differently colored "marbles" together to form pellets.

The simplest form of four-colored filter may be made by placing four pellets of colored wax, or three of colored and one of uncolored, on the convex side of a watch glass in the form of a square surrounding the center, then turning the watch glass upright and pressing it firmly onto a glass plate. The wax serves as readily as a background for direct illumination, as a filter for transmitted light.

The most satisfactory filters for use with higher magnifications were made as follows: Pellets of colored wax were flattened to the desired thickness by being compressed between two glass plates wet with alcohol. The thickness was controlled by two strips of cardboard between the plates. A triangular piece was cut from each flattened pellet and properly spaced around the summit of the convex surface of a small watch glass with the apices directed inward. After the alcohol had evaporated, the watch glass was attached to a glass slip by compressing the wax triangles.—*A. B. Dawson.*

WOLFSON, C. **An improvised inverted microscope.** *J. Lab. & Clin. Med.*, **27**, 809–11. 1942.

An inverted microscope for use in photomicrography, tissue culture and micrurgy may be prepared as follows: Affix the arm and body of a standard microscope (Spencer Model 68 was used) to a cast iron base. Replace the nosepiece with a prism chamber (3¼ in. long with an internal cross section of 1.0 in., made of ⅛ in. sheet aluminum). Screw it into the body tube by means of a threaded collar and lock with a lock nut. Drill two 5/8 in. holes in the top of the chamber, one concentric with the body tube and the other with the objective. Place two right-angled prisms in the chamber, fitting them snugly, and fasten them by screws thru the floor of the chamber and by plates which close the ends. Mount the three objectives on a brass plate, each threaded into a collar. Make the plate movable between two guides perpendicular to the length of the prism chamber. Fit a threaded machined band about the collar and fix it with a set screw. Each band should have a machined depression which receives a spring-backed pin when the objective is centered. Support the condenser and the light source (an 18 v. toy train lamp) on a sturdy upright mounted on the base (a 5½ in. length of ¾ in. square brass rod with a brass plate affixed to its upper end by means of a centered screw). Fix a mechanical stage to the iron base. Use the entire condenser with the rack and pinion (Spencer Model 3 Research Microscope). Fasten a thin upright to the condenser for supporting a cover slip to serve as a stage between the condenser and the objective. This equipment is readily available and is inexpensive.—*John T. Myers.*

PHOTOMICROGRAPHY

KOCH, WALTER. **Increasing the depth of focus in photomicrography by incident light.** *J. Roy. Micr. Soc.*, **61**, 86–7. 1941.

The depth of focus, using incident illumination, can be increased by inserting a stop immediately above the object glass. Three thin circular plates are prepared, each the diameter of the fixed diaphragm of the object glass. In the center of each plate is a hole, 1, 2, and 3 mm. in diameter, respectively. A series of 5 photographs is made of a stage micrometer viewed thru the microscope by transmitted light. The first is made with the micrometer horizontal; the second with one end of the micrometer lifted about 2 cm.; the third, fourth, and fifth are made with the micrometer in the oblique position, each stop being inserted in turn above the object glass. The depth of focus is determined by counting with the aid of a magnifier the number of division marks which appear sharp on each photograph. The narrower the stop the more marks will appear sharp; however, with increased narrowness there is a progressive loss of resolving power.—*C. E. Allen.*

MICROTECHNIC IN GENERAL

BRUG, S. L. **The use of cajuputin oil in microscopy.** *Trans. Amer. Micr. Soc.*, **61**, 84–5. 1942.

Cajuputin oil is a useful substitute for xylol in wet climates because it will absorb small amounts of water without clouding. Apparently it was not tried as a solvent for balsam.—*Virgene Kavanagh.*

ELLINGER, P. **Fluorescence microscopy in biology.** *Biol. Rev. Cambridge Phil. Soc.*, 15, 323–50. 1940.

An historical review of fluorescence microscopy is given, starting with the work of Kohler, in the development of microscopy with ultra-violet light. In a section on technic the principles involved are discussed and the type of apparatus necessary is described. Attention is given to recent advances in this field of technic, especially in regard to new sources of ultra-violet light and to the use of flourescent dyes to increase the visibility of objects in such light. A section on results follows which is divided into the following sub-headings: examination of spontaneous fluorescence of animal and plant tissues; fluorescence microscopy in bacteriology; results of intravital microscopic studies. The article ends with a summary and a good bibliography, thus providing a useful introduction to a study of the field covered.—*H. J. Conn.*

MEDAWAR, P. B. **The rate of penetration of fixatives.** *J. Roy. Micr. Soc.*, 61, 46–57. 1941.

Plasma is prepared from a cockerel by bleeding from the carotid artery, spinning the blood, and storing the supernatant plasma in waxed test tubes on ice. For experimental purposes it is coagulated by warming to 37° C., or by adding a trace of a tissue extract in saline, in short glass tubes of constant bore sealed at one end. These tubes are stood in stoppered vessels containing a volume of fixative at least 30 times the volume of the plasma. The fixative is periodically renewed. For fixatives which precipitate proteins, the rate of fixation is measured by the distance, at definite times, of the surface of the plasma from the boundary formed at the level where the reagent is of a concentration sufficient for precipitation. For other fixatives, special indicators are added to the plasma: for potassium dichromate, methylene blue; for acetic acid, brom thymol blue; for osmic acid, 1% pyrogallic acid; for formaldehyde, phenylhydrazine hydrochloride in saturated aqueous solution; for iodine, starch solution. Temperature and the concentration of the fixative remaining constant, the distance (x) penetrated by the wave front (boundary of precipitation) depends upon the following function of the time of fixation (t):

$$x = K\sqrt{t}$$

K is called the coefficient of penetration. Its value was determined for each of various simple fixatives, in the concentrations in which these are used in standard fixing fluids, as well as for the combined fluids themselves. In the table of results given, K is highest for 25% acetic acid, lowest for 1% tannic acid.—*C. E. Allen.*

PENNY, S. F. **A substitute for cover glasses in mounting pathological specimens.** *Can. J. Med. Techn.*, 2 (No. 2, March). 1940.

Due to rise in price and lack of German supply, the author tried various thicknesses of cellophane, plastocele, and mica, using gum dammar as mounting medium. All proved unsatisfactory. Attempts were made to find a transparent plastic material which could be applied in liquid form, drying with a hard, smooth surface. Clear Duco was fairly satisfactory, but a new quick-drying clear lacquer has proved the best material so far. The lacquer used is C.I.L. Special Slide Lacquer XB 6965–(S.R. 2452; I.R. 23–710). Retail price, January 1940, was $7.50 per gallon, obtained from Paint and Varnish Division, Canadian Industries, Ltd. This lacquer has an advantage over Duco in that it is soluble in xylol, and may be applied to sections direct after removal from xylol. Apply the lacquer by brushing it lightly over the section and the surrounding slide with a camel-hair brush about 1.5 cm. in width. The sections are then allowed to dry at room temperature and are ready to be examined after 15 minutes. The lacquer surface smoothes out and brush marks disappear. It has remained clear after exposure to light for several months. Under high-power oil immersion the cell detail is slightly hazy. The mixture index was estimated to be 1.4835, and this is reputed to be the nearest index to glass of any of the quick-drying lacquers. If a high-power dry objective with compensating collar is used, the refractive error can be corrected and cell detail becomes quite clear.—*S. G. L., in J. Roy. Micr. Soc.*, 61, 105.

PUSEY, M. A. **Methods of reconstruction from microscopic sections.** *J. Roy. Micr. Soc.*, 59, 232–44. 1939.

Drawings are made from a series of sections (not less than 13 μ in thickness) and then superimposed in the original order in a tracing box lighted from above and below, the paper sheets bearing the drawings being made transparent with xylene. At three points in a triangle, "guide holes" are made thru the whole pile of drawings. In preparing a "contour picture", the position of the guide holes is noted on the tracing paper; then, orienting the drawing by means of the guide holes, the outlines of the selected structure or structures are traced from the first drawing. Later drawings in proper order replace the first, and those parts of each drawing are traced which project beyond previous drawings. By appropriate use of dotted and heavy lines the contours of the structure or organ in question are adequately shown. A method is outlined also for securing a "projection reconstruction" at right angles to the planes of the original sections. Many detailed recommendations are offered with regard to the trimming of the block in which material is imbedded, the cutting and mounting of sections, outlining of the sections on the slide with a black paint, and the preparation of drawings (using a microprojector rather than a camera lucida).—*C. E. Allen.*

DYES AND THEIR BIOLOGICAL USES

BARNES, W. A. **Effect of Congo red on plasma prothrombin.** *Proc. Soc. Exp. Biol. & Med.*, 49, 15–9. 1942.

The hemostatic action of Congo red may be explained in some cases by its vitamin-K-like action in raising the level of plasma prothrombin. This action was tested in mice, the level of plasma prothrombin of which had been lowered by feeding mineral oil.—*M. S. Marshall.*

CANNON, H. GRAHAM. **On chlorazol black E and some other new stains** *J. Roy. Micro. Soc.*, 61, 88–94. 1941.

The author introduced chlorazol black E into microscopic work in 1937, and now calls further attention to it, re-emphasizing its special usefulness. He states that in some of his preparations he noticed a faint pink, which proved to be due to another dye present. This dye was isolated for the author by the Imperial Chemical Industries, and he has named it "lignin pink" because of its affinity for lignin in staining plant tissue; it is a mono-azo dye. A useful double stain for plant sections consists of 0.5 g. each of chlorazol black E and of lignin pink in 100 ml. water, with a 20–30 minute staining period.

A further series of new stains has been developed as a result of these studies and are now on sale by the British Drug Houses, Ltd. The author names these dyes as follows: "Hickson purple", a disazo dye of unusual chemical structure; of use in differentiating blood cells in tissues. "Marshall red", a disazo dye belonging to the J-acid urea class of direct dyes; of use in combination with "victoria green G". "Beyer brown", a disazo dye of the benzidine class; a good tissue stain, producing results very similar to Ehrlich's hematoxylin. "Victoria green G", a trisazo dye of the benzidine class of direct cotton dyes; stains distinctly differently in aqueous and in alcoholic solution, and can give good double staining effects with "Hickson purple" or "Marshall red", altho these dyes are all essentially nuclear stains. "Manchester blue", a disazo direct cotton dye of the benzidine class. "Owen's blue", a dye similar in constitution to the last mentioned; a very powerful stain with considerable metachromatic effect on plant tissue.

The double staining methods recommended call for 20 min. in saturated aqueous Marshall red or 10 min. in saturated aqueous Hickson purple, followed by 30–60 min. in saturated Victoria green G in 70% alcohol, with a rinse in 90% alcohol, dehydration and mounting. Either combination gives good double staining in animal tissue.—*H. J. Conn.*

CRAIG, JOHN J., and CASS, W. E. **Derivatives of aminoisoquinolines.** *J. Amer. Chem. Soc.*, 64, 783. 1942.

The procedures for the preparation of several compounds from isoquinoline and preliminary pharmacological tests on the sulfanilyl derivatives are described. The compounds prepared are 4-bromo-, 4-amino-, and 5-aminoisoquinolines and

acetyl, benzoyl, acetylsulfanilyl and sulfanilyl derivatives of 1-, 4-, and 5-aminoisoquinolines. Results of the pharmacological tests show that 5-sulfanilamidoisoquinoline exhibits toxic symptoms in mice in dose levels of 5, 10 and 20 mg. per 20 g. of animal body weight, while the 1-, and 4-sulfanilamido derivatives do not. In tests for antistreptococcal efficacy in mice, protection was obtained with 1-sulfanilamidoisoquinoline comparable with a sulfadiazine standard; protection by the 4- derivative was somewhat less; the 5- derivative was ineffective.—*A. P. Bradshaw.*

DAVIS, EDWIN. **Aniline (triphenylmethane) dyes in the treatment of Hunner ulcer, preliminary report.** *J. Urol.*, **46**, 899. 1941.

The alkylated triamino-triphenylmethane dyes, gentian violet, methyl violet, ethyl violet and crystal violet, were found to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* in dilutions of 1:10,000 to 1:1,000,000 in urine of pH 5.5 and 7.5. These dyes were usually inhibitory at higher dilutions in urine of pH 7.5 than of pH 5.5.

For the clinical trials, purified methyl violet B (American Aniline Products Company) and crystal violet (Du Pont) were mainly used. For installation, the concentrations used were 1:100 to 1:20,000, while for irrigation, they were 1:10,000 to 1:20,000. Local treatment of the submucous ulcer with these dyes usually resulted in the following sequence of events: an irritating reaction of 1 to 10 days duration, a period of relief averaging 5½ months and then a relapse.—*L. Farber.*

FREDERICK, WILLIAM G. **Estimation of small amounts of antimony with rhodamine B.** *Ind. Eng. Chem., Anal. Ed.*, **13**, 922. 1941.

This paper reports the result of an investigation of the reaction between quintivalent antimony and rhodamine B, and describes a colorimetric procedure for the determination of antimony encountered in tissue and air, based upon this reaction. Rhodamine B reacts with quintivalent antimony in hydrochloric acid solution or sulfuric acid solution containing chloride ion, to form a red, water-insoluble stable compound, soluble in organic solvents. The excess reagent is destroyed with bromine, the rhodamine B complex put into solution by addition of alcohol, and the amount present estimated colorimetrically in a Duboscq colorimeter. Practically all acid radicals except chloride and sulfate interfere, as do arsenic, iron, and certain metals of the dithizone group. The latter interference can be avoided by preliminary extraction with dithizone. The method will detect as little as 0.1 γ of quintivalent antimony in 5 ml. of solution, and is rapid, inexpensive and specific. The extract procedure is described for the concentration range 0.1 to 300 γ of antimony. (There are many precautions given in the original article and it would be advisable to refer to it before attempting the estimation of antimony by this method.)—*A. P. Bradshaw.*

GREEN, J. H. **Sodium salt of quinizarin-6-sulfonic acid as an acid-base indicator.** *Ind. and Eng. Chem., Anal. Ed.*, **14**, 249. 1942.

This indicator can be used for colorimetric determinations of pH over the range from 7 to 11.5 in the titration of strong acids with strong bases, or of weak acids with strong bases. The indicator has two color changes, yellow to pink (pH 7.1–9.1) which is sharp, and pink to blue-violet (pH 9.1–11.5) which is gradual. The author found that in titrating acids with bases the color change was sharper when using the sodium salt of quinizarin-6-sulfonic acid than when using phenolphthalein, and in titrating bases with acids the quinizarin indicator gave a sharp color change, whereas phenolphthalein could not be used so efficiently.—*A. P. Bradshaw.*

HENDERSON, H. J., and LONG, E. R. **Accumulation of chlorophyll pigments in visceral organs and their elimination.** *Proc. Soc. Exp. Biol. & Med.*, **48**, 438–9. 1941.

Chlorin-e and rhodin-g were eliminated slowly from the tissue of rabbits after intravenous injection. Most of that deposited in the spleen and bone marrow during a month of inoculations was still present in phagocytic cells three months later. Elimination from the liver was somewhat greater. The deposit in hemopoietic organs had no evident effect on their function.—*M. S. Marshall.*

LEHR, DAVID, and ANTOPOL, WILLIAM. **Specific morphology of crystals appearing in the urine during administration of sulfanilamide derivatives.** *Amer. J. Clin. Path.*, 12, 200–9. 1942.

Sulfanilamide derivatives are excreted in the urine as "impure" crystals with the following specific morphology: Sulfathiazole crystals are amber green and are shaped like dumb-bells, rosettes or hexagonal platelets. Sulfadiazine is excreted in acetylated form with crystals of amber green shells and excentrically bound shocks of wheat; or in free form, with dark green globular and chestnut burr crystals. Sulfapyridine crystals resemble whetstones, arrowheads or coarse, centrally waisted sheaves. Sulfaguanidine crystals appear as oblong rectangular plates bulging slightly in their long axis.—*H. Gordon.*

MICHELSON, LOUIS. **An improved polychrome methylene blue eosinate.** *J. Lab. & Clin. Med.*, 27, 551–3. 1942.

The stain is prepared as follows: Dissolve 1 g. of methylene blue chloride (certified) in 100 cc. of *N*/100 NaOH (carbonate free) in a 500 cc. glass-stoppered bottle. Heat in a water bath for 2½ hr. at 55° C. Shake vigorously for 1 min. at half-hour intervals. Add 1 g. of NaBr, heat again for 2½ hr., cool and filter. Add 60 cc. of 1% aqueous eosin Y and mix with a stirring rod. Add more eosin in 5 cc. amounts till the solution has a reddish tinge. Let stand 18–24 hr. and filter. Dry completely at 37° C. Store in a glass-stoppered bottle. Dissolve 0.1 g. in 600 cc. of absolute acetone-free methyl alcohol. Let stand 2–3 days and filter.—*John T. Myers.*

ROBLIN, RICHARD O., JR., WINNEK, PHILIP S., and ENGLISH, JACKSON P. **Studies in chemotherapy. IV. Sulfanilamidopyrimidines.** *J. Amer. Chem. Soc.*, 64, 567. 1942.

The preparation and properties of a series of sulfanilamidopyrimidines related to sulfadiazine is reported. With the exception of 2-sulfanilamido-4, 6-dimethylpyridine, none of the compounds in this series showed the same high degree of absorption and chemotherapeutic activity against experimental streptococcal and pneumococcal infections, as sulfadiazine. Because of the unfavorable influence of a methyl group on toxicity as evidenced by sulfamethylthiazole, an extensive investigation of toxic properties would be required before a true evaluation of its utility could be given. 5-Sulfanilamidopyrimidine was active but was not as effective as sulfadiazole. The 2-amino derivative was found to be only slightly active. When the sodium salt of this compound was administered subcutaneously it produced a much better therapeutic effect by increasing the blood levels. 2-Sulfanilamido-4-methoxypyrimidine was less active than the corresponding 4-methyl compound.

4-Sulfanilamidopyrimidine was described as inactive on the basis of *in-vivo* experiments published in a previous paper. 2-Sulfanilamidopyridine was found active against experimental infections in white mice. Since such a marked distinction between the 2- and 4-isomers seemed unlikely, the 4-isomer was retested *in vitro* and found to show as great a bacteriostatic effect as sulfadiazine. The authors call attention to pitfalls which might be involved in the use of *in vivo* data as a basis for attempts to correlate chemical structure with chemotherapeutic activity.—*A. P. Bradshaw.*

WHITBY, G. S. **Dyes and dye chemistry fight disease.** *Textile Colorist*, 64, 119. 1942.

This interesting review brings out the historical development of chemotherapy and the direct discovery of better colorless drugs for use as chemotherapeutic agents arising from the study of the use of dyes for this purpose. Trypan red, in the treatment of the special disease Mal de caderas, led ultimately to (a) the colorless remedy Germanin, for human sleeping sickness, (b) the arsenical drugs, such as Salvarsan for syphilis. From Prontosil, a dyestuff by virtue of its azo group, the new class of synthetic drugs, the sulphonamides, has been developed. Furthermore, leading to the discovery of Prontosil was (a) the discovery that the yellow azodyl, chrysoidin, had appreciable antiseptic powers *in vitro*, altho lacking *in vivo*; (b) the discovery of the bacterial properties of certain sulphonamides; and (c) the successful employment as an internal antiseptic of an azo dye, pyridium.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

ANONYMOUS. **Hastings' Stain. Method of Preparation.** *J. Amer. Med. Assoc.*, **118**, 936. 1942.

In answer to a query concerning the preparation of Hastings' stain for blood films in determining the opsonic index in undulant fever, the modification of Wright's stain known as "Hastings' stain" for blood films is described as follows: "It was described by T. W. Hastings (*J. Exp. Med.*, **7**, 265, 1905) and differs from the stain described by Wright in that the polychrome methylene blue is neutralized with acetic acid and, when mixed with the eosin solution, a small amount of fresh methylene blue solution is mixed with it." I. F. Huddleson specifies this stain in a recent publication and says that it can be purchased from Hartman-Leddon Co., Philadelphia, the only reliable source he has found. There is no information available to the Biological Stain Commission to indicate that there is any essential difference between this stain and the one this company sells as Wright's stain.—*Elizabeth F. Genung.*

BAKER, JOHN R. **A fluid for softening tissues embedded in paraffin wax.** *J. Roy. Micr. Soc.*, **61**, 75–8. 1941.

A mixture of 9 parts of 60% alcohol and 1 part of glycerin is recommended for mammalian liver, pancreas, kidney, and other tissues. The block containing the imbedded material is soaked in the softening fluid after a surface of the tissue is fully exposed by a razor cut. Rate of penetration varies with the tissue and with the fixative used. Soaking overnight is generally satisfactory.—*C. E. Allen.*

COOKE, JEAN V., and BLATTNER, R. T. **Trypan-blue vital staining in studies of virus lesions on chorio-allantoic membranes.** *Amer. J. Path.*, **18**, 163–7. 1942.

The authors describe the following new application of trypan-blue for vital staining in which virus foci grown on chick chorio-allantoic membranes are selectively outlined by the dye: When the membrane is ready for examination, following inoculation and incubation, place 1 cc. of a 0.5% aqueous suspension of trypan-blue (dye content and source not stated) directly upon the membrane thru the window in the shell. It was found that the stain could be best applied to the membrane with a No. 15 DeVilbiss atomizer. After application of the stain close the shell window and place the egg in the incubator for 10 to 30 min., the incubation time depending upon the age and extent of the lesion. At the end of the period of incubation remove the membrane in the usual manner, wash gently in physiological saline to remove the excess of trypan-blue, and fix flat in a 4% solution of formaldehyde for a few minutes. After fixation drain the excess formalin from the membrane and mount in warm glycerin jelly on a large glass slide. For mounting, melt the glycerin jelly and drop it on the membrane until the latter is covered. Flame the slide gently and add a warm cover slip, applying gentle pressure to the cover slip until the jelly sets. When hardened, remove the excess jelly from around the edges of the cover slip and seal the edges with clear Duco cement thinned with acetone.—*J. O. Foley.*

FETTERMAN, GEORGE H. **A simple and reliable method of staining spermatozoa.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 9. 1942.

Abnormal spermatozoa can be identified in smears of fresh seminal fluid by the following method: Air-dry the smear; fix 3 min. in 10% aqueous formalin (4% formaldehyde); stain 1 min. with Harris hematoxylin; wash; dry.—*H. Gordon.*

HARTZ, PHILIP H. **Routine staining of the beta cells of the islets of Langerhans with Masson's tetrachrome stain.** *Arch. Path.*, **33**, 541–2. 1942.

The β cells of pancreatic islet tissue may be stained specifically by the following method: Fix thin slices of pancreatic tissue 12–24 hr. in equal parts Bouin's fluid and saturated aqueous solution of $HgCl_2$; embed in paraffin; cut 6 μ thick; deparaffinize; remove mercury precipitate; stain in Larson and Levin's modifica-

tion of Masson's tetrachrome stain (*Arch. Path.*, 29, 272, 1940), substituting light green with fast green; dehydrate; clear; mount in balsam. Result: the β cells contain red granules.—*H. Gordon.*

HASS, GEORGE M. **Studies of Cartilage. III. A new histochemical reaction with high specificity for cartilage cells.** *Arch. Path.*, 33, 174–81. 1942.

Cartilage cells react in a specific manner to the following technic: Section fresh blocks of cartilage at 15–20 μ on the freezing microtome; wash; place for 45 min., at room temperature in a mixture of 10 ml. 0.5 molar HCl, 4 ml. 0.04 molar crystal violet, and 1 ml. 0.2 molar $NaNO_2$; add 7–8 ml. 0.5 molar NaOH, till the solution becomes slightly fluorescent and forms a slight precipitate after 10–20 min.; pour ether over surface of solution; withdraw and wash sections; float on slide; blot with filter paper; stain with hematoxylin; mount in glycerogelatin.

Results: a positive reaction is indicated by aggregations of spherical or discoid, refractile yellow granules, 1–3 μ in diameter, occurring in the cytoplasm of cartilage cells. The nature of the reacting cytoplasmic component is unknown. Infantile cartilage reacts more uniformly than adult cartilage. Noncartilaginous tissues do not react.—*H. Gordon.*

HOLMES, WILLIAM. **A new method for the impregnation of nerve axons in mounted paraffin sections.** *J. Path. & Bact.*, 54, 132–6. 1942.

The method is applicable after formalin fixation and uses $AgNO_3$ as the impregnating agent. It gives a uniform impregnation of the axons of medullated and non-medullated nerve fibers, and in preliminary tests has given good results with material from the central nervous system.

The procedure is as follows:

Fix specimens upon removal in a mixture of 40% neutralized formalin, 15 cc., and 0.8% aqueous NaCl, 85 cc. Transfer tissue directly to 95% alcohol, pass thru absolute alcohol, and prepare paraffin sections as usual. Attach sections to albuminized slides, dry 24 hr. at 37°; 15 μ is a good thickness if no counterstain is to be applied. Soak the slides for 48 hr. at 37° C. in a mixture of xylene, 30 cc., and glacial acetic acid, 70 cc. Take down to distilled water thru graded alcohols. Impregnate with a mixture of 0.01% aqueous $AgNO_3$, 100 cc., and concentrated ammonia, one drop for 5–24 hr. at 37° C. This solution can be used only once. In subsequent stages the slides are treated individually. Rinse in distilled water for 5 sec. Reduce in either a strong or weak reagent. Preparation of the strong reducer is as follows: anhydrous Na_2SO_3, 5 g. (or 10 g. of hydrated); $NaHSO_3$, 2.5 g.; water, 100 cc. To this add immediately before use 0.5 g. amidol. To prepare weak reducer: dissolve 1 g. hydroquinone, 5 g. anhydrous Na_2SO_3, in 100 cc. water. Place slides for 30 sec. in the reducer. Wash slides by violent agitation in running water 10–15 sec., then in distilled water 5 sec. Tone sections for 3–5 min. in 0.2% of either yellow or brown gold chloride. If the yellow salt is used, add 4 drops of glacial acetic to each 100 cc. of solution. Agitate slides 5 sec. in distilled water. Reduce in 2% aqueous oxalic acid for not more than 10 min. Control appearance of nerve fibers under the microscope. Rinse slides briefly in distilled water. Place in 5% aqueous $Na_2S_2O_4$ for 5 min. If the solution becomes cloudy the oxalic acid was not removed by the previous rinse. Wash slides thoroly in distilled water; counterstain if required; clear and mount.

The stronger reducer brings out finer axons but stains other structures deeper. Preliminary results indicate that the method may be applicable to all parts of the nervous system and to material fixed in other fixatives.—*S. H. Hutner.*

KOLIN, MORRIS. **Technic for sectioning soft bones and hard tissues by celloidin and paraffin methods.** *Arch. Path.*, 33, 86–7. 1942.

The staining qualities of soft bones and hard tissues are protected and their sectioning made easy by the following technic: Fix 24 hr. in 1:9 aqueous formalin (4% formaldehyde); wash 3–4 hr.; soften 24 hr. in 5% aqueous glacial acetic acid (C. P.); wash 24 hr.

For celloidin embedding: dehydrate 1 hr. in 80% alcohol; 1 hr. in acetone (C. P.); 3–6 hr. in oil of clove (U. S. P.); infiltrate 24–48 hr. with 8% pyroxylin in equal parts ether and absolute alcohol; embed in 12% pyroxylin.

For paraffin embedding: dehydrate 12 hr. each in 80%, 95%, and absolute alcohol; infiltrate 1½ hr. in chloroform; 2 hr. at 52° C. in chloroform, 1 part, with paraffin, 2 parts; 2 hr. in pure melted paraffin; embed in paraffin.

Routine hematoxylin (Harris) and eosin staining methods are recommended after both types of embedding.—*H. Gordon.*

PEARSON, GRACE A. **Eosin Y for tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 16, 1942.

A rich, fast-staining eosin Y may be obtained as follows: Dissolve 2.5 g. eosin Y (National Aniline and Chemical Co.), in 500 ml. water; add, drop by drop, 4 cc. concentrated HCl; allow precipitate to settle; decant supernatant fluid; wash precipitate 6–8 times in 500 ml. distilled water; filter; dry in oven; dissolve in 200 ml. 95% alcohol; use full strength. Protect the dye by immersing slides in 95% alcohol before staining in the eosin.—*H. Gordon.*

RITCHIE, GORTON. **A simple iron hematoxylin method for tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 10. 1942.

Designed to fortify the action of weak hematoxylin for Van Gieson's stain, the method is as follows: Mordant sections 10 min. in 2–4% aqueous $FeNH_4(SO_4)_2$ + 12 H_2O (violet crystals); wash well in tap water; stain 2–5 min. with hematoxylin (Harris or Delafield).—*H. Gordon.*

STOVALL, W. D., and PESSIN, S. B. **Problems in the laboratory diagnosis of rabies.** *Amer. J. Pub. Health*, **32**, 171–4. 1941.

The authors find eosin-methylene-blue the most satisfactory stain for demonstrating Negri bodies in routine diagnostic work, both for paraffin sections and for smear preparations. It is important that staining solutions of low pH be used. A 1% eosin Y in 95% alcohol maintained at pH 6 stains Negri bodies a pale pink color; at pH 3 the large and small Negri bodies stain a uniform deep red. The counterstain is methylene blue adjusted between pH 5.0 and 6.0. When eosin and methylene blue are maintained at the optimum pH, the cytoplasm of the nerve cells takes a diffuse pale blue color and the body of both the small and large Negri bodies a deep red. The inner structure of the Negri bodies appears as dark blue granules. The "lyssa bodies" are a homogeneous red color.—*M. W. Jennison.*

WILSON, WILLIAM L. **A quick easy method of preparing tissues for microscopic examination by combining the technics of Nelson and Terry.** *J. Lab. & Clin. Med.*, **27**, 537–42. 1942.

The following method is quick, easy and inexpensive: Dissolve 3 g. of crystalline egg albumin in 10 cc. of tap water. Place a cellophane container in a dish or beaker and put the block of tissue in it. Fill the cellophane container with albumin solution and pour the fixing fluid (40% formaldehyde) around it. Let stand 2–8 hr. depending on the temperature (140–170° F.). Hold the block in running water for a few seconds, and peel off the cellophane, stain with Terry's neutralized polychrome methylene blue; mount and examine. This technic is particularly useful in the office of surgeons and gynecologists.—*John T. Myers.*

PLANT MICROTECHNIC

BHADURI, P. N. **Improved smear methods for rapid double staining.** *J. Roy. Micr. Soc.*, **60**, 1–7. 1940.

The following changes are suggested in the Feulgen-light-green method previously described: For root-tip smears, treat roots 3–24 hr. (according to species) with 0.1% colchicine or 1% chloral hydrate. Fix tips in Levitsky's fixative (6:4) for 2–3 hr.; rinse in water; soak 5 min. in warm water; hydrolyze in *N* HCl 20–30 min. at 60° C.; and stain in decolorized basic fuchsin solution 20–30 min. Smear the tissue in 45% acetic acid between cover glass and slide; remove the cover glass in acetic alcohol (1:1), and leave the smear in this solution 10 min. Then pass thru 2 changes of 80% alcohol, 5 min. each. Mordant for 45–60 min. in a clear saturated solution of Na_2CO_3 in 80% alcohol, wash in 3 changes, 5 min.

each, of 70% alcohol, bring to 90% alcohol and stain 10–15 min. in light green. Differentiate the stain in 80% alcohol with a trace of Na_2CO_3, followed by 90%, 95%, and absolute alcohol, and clear in graded xylol-alcohol. Mount in "Sira" medium.

A more rapid method for general cytological observation is outlined, involving fixation in modified Carnoy (3:1:1) and omitting the light green stain. Modifications are suggested for preparations of pollen mother cells and pollen grains.—*C. E. Allen.*

CHEN, H. K., and THORNTON, H. G. **The structure of "ineffective" nodules and its influence on nitrogen fixation.** *Proc. Roy. Soc.* (*B*), **129**, 208–29. 1940.

For studying distribution of starch, material was fixed in Allen's modification of Bouin's solution (Allen's P.F.A. 3) and occasionally in Flemming's (McCoy 1929). Slides were treated with a mordant of 2% aqueous solution of tannin for 12 hr., stained 2 min. with 1% aqueous crystal violet and differentiated in alcohol.—*T. E. Weier.*

MICROORGANISMS

HAITINGER, M., and SCHWERTNER, R. **Beiträge zur Fluoreszenzmikroskopie in der Bakteriologie.** *Zentbl. Bakt. I Abt. Orig.*, **145**, 141–4. 1940.

Smears of sputum and pus gave satisfactory results with the fluorescent microscope when the following technic was used:

Fix smears with heat; flood with 1:500 aqueous acridin for 30 sec.; wash quickly; differentiate 10–15 sec. with ethyl alcohol containing 4% HCl; wash and dry in air or with heat. Bacteria stain a golden color in a midnight-blue field.—*H. J. Peppler.*

HANNA, G. D., and GRANT, W. M. **Preliminary note on a technique for mounting diatoms in realgar and other substances.** *J. Roy. Micr. Soc.*, **59**, 174–6. 1939.

Exceedingly stable films of realgar, selenium, or tellurium can be produced by evaporation in a vacuum. The same is true of arsenic, which however seems to adhere less tightly. Standard apparatus used in the aluminizing of mirrors for microscope illuminators is employed; the tungsten filament commonly used for aluminum is replaced by a short band of nichrome ribbon. A minute amount of the material to be evaporated is placed on a thin sheet of mica, which is rested on the heater. The cover slips bearing strewn diatoms are attached to a lantern-slide cover plate, and this is suspended, diatoms facing downward, a few inches above the heater. Evaporation requires but a few seconds. Covers so treated are attached to slides with hyrax and surrounded by rings of shellac. Resolution is as satisfactory if the diatoms are coated with a half wave-length of the material as if the thickness equals two or three wave-lengths.—*C. E. Allen.*

HOLLBORN, K. **Ein verbesserter Gram-Simultanfarbstoff.** *Zentbl. Bakt., I Abt. Orig.*, **143**, 160. 1939.

The victoria-blue-pyronin technic for Gram's staining procedure (*Zentbl. Bakt. I Abt. Orig.*, **136**, 506, 1936) has been improved and now can be applied to smears in routine examination for gonococci. The following procedure has been suggested: flood extremely thin smears of pus with the staining solution for 1 min.; wash in distilled water; blot with filter paper; dry in air. The dye mixture for the simultaneous Gram procedure is obtainable only from the author (K. Hollborn and Söhne. Leipzig).—*H. J. Peppler.*

KNAYSI, GEORGES. **The demonstration of a nucleus in the cell of a staphylococcus.** *J. Bact.*, **43**, 865–85. 1942.

Several chemical reactions have been adapted to demonstrate the nuclear nature of a central granule in a staphylococcus. These are as follows:

1. Stain dried flame-fixed smears with methylene blue solutions of varying pH (to 5 ml. of 1% KH_2PO_4 add 0.1 ml. distilled water, 0.1 ml. *N* HCl, or 0.2 ml. *N* HCl and then 0.1 ml. 1% aqueous methylene blue, to each) while observing

under the microscope. The nucleus stains more deeply than the cytoplasm at the lower pH values. It may be necessary to decolorize with 1% H_2SO_4 to see the nucleus of a rapidly growing cell.

2. Stain dried flame-fixed smear with Meyer's methylene blue (1 ml. saturated alcoholic methylene blue diluted with 10 ml. of water) for 2 min.; drain the slide and place it in 1% aqueous methylene blue containing 1 or 2% acetic acid for 3 min.; rinse thoroly in running water; blot; dry; and examine.

3. Millon's test for proteins. Flood the slide with Millon's reagent (1 ml. mercury in 9 ml. HNO_3, sp. gr. 1.420 to 1.425, diluted with twice as much water) and heat until steam is visible several times; rinse with running water; remove the white precipitate by immersing the slide in 1% HNO_3; rinse again; blot; dry; examine in a drop of water under a cover glass. The nuclear protein gives a reddish brown color.

4. Sharp's test for proteins. Place the air-dried flame-fixed smear in 5% glucose at 100° C. for 24 hr.; rinse thoroly in running water; dry; examine for dark brown protein particles which appear to be stained with colloidal silver; place a drop of 10% NaOH on the smear under the cover glass and seal it in place; the color is removed slowly.—*Virgene Kavanagh.*

LIND, HOWARD E., and SHAUGHNESSY, HOWARD J. **Fluorescent staining technic for the detection of acid fast bacilli.** *J. Lab. and Clin. Med.*, **27**, 531–6. 1942.

The flourescent technic used was as follows: Apply auramin solution (0.1 g. auramin, National Aniline Co., and 57 cc. distilled water) for 2 min. Wash and decolorize (70% alcohol 100 cc., concentrated HCl 0.5 cc., and NaCl 0.5 g.) for 2–4 min.; then decolorize for 2 min. more in fresh decolorizing solution. Wash, dry and examine. The necessary special equipment may be obtained from the Spencer Lens Co. Place the blue ultraviolet filter on the lamp in front of the light, replace the mirror by one coated with aluminum and focus as much light as possible into the draw tube. An 8 mm. objective and 20× ocular are recommended. This gives a field 20 times that of the usual oil immersion field, with a magnification of 400. The acid-fast bacilli appear as small, bright yellow rods against a dark background. The fluorescent method proved to be more sensitive than the Ziehl-Neelsen technic even tho the specimens of sputum being examined contained tricresol which produces fluorescence and interferes with the examination. The disadvantages of the fluorescent technic are the use of a monocular microscope, the interference by tricresol, and possible greater fatigue. These are counterbalanced by the advantages of larger fields, simplicity, rapidity, and increase in the number of positives.—*John T. Myers.*

MANER, R. **Über die Färbung der Spirochaeta icterohaemorrhagiae und anderer Leptospiren.** *Zentbl. Bakt., I Abt. Orig.*, **143**, 462–4. 1939.

Twenty different dyes commonly used in staining parasites failed to reveal selected spirochaetes as colored or colorless bodies. Smears fixed with methyl and ethyl alcohol, or a mixture of alcohol and ether (no ratio given), or osmium vapor and stained with Giemsa's solution were most satisfactory for practical purposes.—*H. J. Peppler.*

NERI, F. **Beobachtungen über den Geiszelapparat der Bakterien (mit einer neuen Färbungsmethode der Bakteriengeiszeln).** *Zentbl. Bakt., I Abt. Orig.*, **146**, 166–74. 1940.

The curled flagella of certain strains of *Salmonella typhi-suis* were best demonstrated by the following method:

Fixing and mordant: (a) saturated $HgCl_2$ (10–15% hot solution); (b) 20% tannic acid + 37 ml. saturated $KAl(SO_4)_2+12H_2O$ (15% hot solution filtered after cooling); (c) 10% $FeNH_4(SO_4)_2+12H_2O$ in cold distilled water. Mix a, b, c (2:1:1); filter before use. (The mixture is good for 2–3 hr.)

Place air-dry slides of 18–24 hr. culture inverted in a watch glass; add mordant until slide floats, and allow to stand for 1 hr.; rinse with distilled water; stain (0.5 g. crystal violet + 10 ml. ethyl alcohol + 90 ml. anilin water) several minutes with heat.—*H. J. Peppler.*

SERLIN, N. J., and LISA, J. R. **A concentration technic for gametocytes of estivo-autumnal malaria.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 8. 1942.

The following method, based on the fact that erythrocytes containing plasmodia have a lower specific gravity than normal red cells, is recommended for examination of blood containing only gametocytes: Evaporate completely 1 ml. of 1% aqueous potassium oxalate in a 15 cc. centrifuge tube; add 5 ml. blood; mix carefully; centrifuge ½ hr. at 2,500 R/M; discard all but ⅛ in. of supernatant plasma; apply one drop of the buffy layer to a glass slide and spread; stain by Wright's method.—*H. Gordon.*

STEUER, W., and SCHINDLER, E. **Über die Leistungsfähigkeit des Brilliantgrünphenolrotagars zur Typhusparatyphusdiagnose.** *Zentbl. Bakt., I Abt. Orig.*, **146**, 49–53. 1940.

A comparative study of the usefulness of Endo agar and brilliant-green-phenol-red agar for the identification of *Salmonella sp.* and *Eberthella sp.* in stools and urine was made with 4053 samples. The brilliant green medium was distinctly superior to Endo agar, showing a marked inhibition of *Proteus sp.*, *Escherichia coli*, and *Shigella sp.* Numerous factors were found to influence the effectiveness of the medium. The following method of preparation of brilliant-green-phenol-red agar is recommended: beef infusion (quantity not stated), 1% peptone, 0.5% NaCl, 1.5% lactose, 4.0 ml. of 0.2% phenol red (to the liter?), 1.0 ml. of 0.5% aqueous brilliant green (to the liter?), 2.5% agar; reaction adjusted to pH 6.8.—*H. J. Peppler.*

WEISS, CHARLES. **An improved technic for the isolation of Bact. granulosis (Noguchia granulosis).** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 19–21. 1942.

Pure cultures of *N. granulosis* may be isolated by the following method: Dilute 1 part Noguchi's leptospira semi-solid medium with 2 parts water; mix 0.5 ml. with an equal volume of 1:5000 aqueous gentian violet or Victoria blue; inoculate medium with 0.02 ml. of suspension; incubate 15 min.; streak on Noguchi's solid leptospira medium; incubate 1 week or longer. The dye inhibits the growth of staphylococci, without affecting *N. granulosis.*—*H. Gordon.*

STAIN TECHNOLOGY

VOLUME 17 OCTOBER, 1942 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

ORCEIN AND LITMUS

It has been remarked more than once in these pages that the present situation in regard to availability of stains is much better than it was during the first World War. The chief exception to this statement has been in the case of the stain, orcein, and the indicator, litmus, both of which have ordinarily been prepared from natural rather than synthetic sources. They have been derived from certain lichens which grow in the tropics, and which yield a product known as archil extract that was a well recognized article of commerce before the present war began. It was prepared in Holland and has not been available since the German occupation of that country; as a result, both orcein and litmus have disappeared from the American market.

When this situation was appreciated, a search was made to see if the lichens could be obtained from somewhere and the archil extract prepared in this country. It was soon concluded that under present conditions this possibility was not promising. More promising, in fact, appeared to be the preparation of the desired stain and indicator by synthetic means. This was undertaken, for the Stain Commission, by MacAndrews & Forbes of Camden, New Jersey. At present, their efforts to produce orcein have been entirely successful, while those to produce a substitute for litmus give promise of success in the near future.

Synthetic orcein, thus prepared, has now been certified by the Stain Commission and is on the market. It can be obtained not only from the manufacturers but from the regular dealers in stains. One of the chief uses of orcein is as a stain for elastin; and the synthetic product has been tested for this purpose with results that are more than encouraging. In fact, those who have employed it as an elastin stain in pathological work have pronounced it much superior to any natural orcein they have ever tried. Another use of orcein is in cytology, and it is not yet entirely sure whether the synthetic product is equal to the natural product for this purpose. It proves difficult to standardize this stain for cytology because it is hard now to find cytologists experienced in the use of this dye who have time available

to assist in the standardization. Accordingly, the suggestion is hereby made that if anyone reading this article is experienced in such a use for orcein he contact the Chairman of the Stain Commission to see if he can assist in this further standardization. The manufacturers as well as the Stain Commission are very anxious to see complete standardization of the dye accomplished.

In spite of this one point which remains to be investigated, it can be said confidently that a splendid synthetic orcein is now available and that the production of a synthetic substitute for litmus is probably just around the corner.

A Manual on Staining Procedures

It has long been a desire of the Stain Commission to formulate the most common staining procedures in standardized form. A beginning in this line has been made by including in the book, Biological Stains, a certain number of such methods; the list of procedures there included, however, is very incomplete. It has proved a slow undertaking to put the various procedures in standardized form, to check them as to their superiority in actual use, and finally to learn the authors of recommended modifications. Whenever, as often happens, indefiniteness and inaccuracy is found in the literature, it is necessary to discover, if possible, what the original author specified, or if that cannot be learned, to find what interpretation to give to the author's statement in order to secure best results. All this takes time.

Nevertheless, the project is now well under way. Coöperation of the members of the Stain Commission has been obtained, and many of the methods are now virtually in shape for publication. It is planned to publish them in the form of a loose-leaf manual, consisting of several leaflets, each dealing with a different phase of microtechnic, that can be revised separately to keep them up to date. The first leaflet to be finished will deal with methods for staining general animal tissue; and plans will be made to put it on sale immediately without waiting for the rest of the manual to be ready. It will probably be published about the first of the coming year; and a notice concerning it will be issued at that time.

In getting out this manual of staining procedures, the Stain Commission has no thought of standardizing *technic*. It is planned to put the procedures in standardized *form*, but not to imply that the methods themselves are standard or "official". They will merely represent the best procedures known at the date of publication, to the body of biologists who make up the Stain Commission. As such they will presumably have more value than procedures that have the experience of only an individual author behind them; but no one is to feel that he must follow them in preference to others that may prove better adapted to his purpose or to his working conditions. In fact, it is proposed to modify these methods from time to time in future editions of the various leaflets, as experience suggests improvements.—H. J. Conn.

IMPROVED CYTOLOGICAL METHODS WITH CRYSTAL VIOLET

C. S. SEMMENS, *King's College, London University, at Bristol, England*

It is well known that the crystal-violet-iodine technic usually provides excellent cytological preparations, the necessary skill for making such preparations is not difficult to acquire and critical examination of detail under the high powers of the microscope is generally possible. It is frequently complained, however, that the stain is not permanent and tends to fade more or less rapidly according to the exact details of the procedure followed. During the past ten years the author has tested many different brands of gentian violet, methyl violet, crystal violet and the related series of dyes in connection with experiments on the chemistry of chromatin, and certain points have been observed that might prove of service in ordinary staining technics.

It has been found that the following method of making up the dye solution leads to better quality preparations which also appear to be quite fast:

Shake up 10 g. of ordinary crystal violet (C.I. 681)[1] with 100 cc. of ethyl alcohol; filter; discard the precipitate; concentrate the filtrate by gentle heat to about 10 cc.; then allow to dry completely at room temperature. Dissolve 1 g. of the extract so obtained in 100 cc. of distilled water and use the solution in the ordinary way.

Preparations stained in the solution made in this way have not shown signs of fading after several years in use for class purposes. Sodium uranate used in the fixative improves the staining and increases the fastness. Clove oil should, of course, be thoroly washed out, and a neutral mounting medium is preferable.

The following technic for staining chromosomes with crystal violet is preferred by the author when cytological detail is required:

Fix root-tip material in Levitsky's chrom-formalin mixture,

[1]The dyes used in the development of the technic were obtained from the following sources: G. T. Gurr, 136 New King's Road, London S.W.6.; British Drug Houses, Ltd. Graham Street, London N.1.; G. Grübler & Co.; National Aniline & Chemical Co. Inc., 40 Rector Street, New York. While the histological and cytological staining effect of the dyes from these sources generally differed considerably, the behavior of the absolute-alcohol extract proved to be fairly uniform and independent of source. The yield of alcohol-soluble fraction varied with both source and batch. In my opinion the dyes supplied by G. T. Gurr and Nat. Aniline and Chemical Co. yielded the best results.

meiotic material in Semmen's sodium uranate fixative.[2] Wash in the usual way and run up to wax thru chloroform. Attach sections to the slide and take down to 75% alcohol; leave in this grade overnight to harden. Run down to water; transfer to warm water, then to *N* HCl at 60° C.; keep at this temperature for 20–30 minutes to hydrolyse lightly. Cool quickly by standing the jar in cold water; rinse with cold *N* HCl, then with water. Transfer to 1% aqueous crystal violet solution made as described above; leave for 1 hour or more; then rinse with water to remove the bulk of the adhering dye; rinse in 75% alcohol followed by 90% alcohol in which the last traces of unlinked dye dissolve out. Dehydrate in absolute, clear in xylol and mount as usual.

The dye becomes firmly linked to the chromosomes and the resulting preparations seem to be as stable and as fast as with the usual Feulgen staining. Differentiation in the alcohol is rapid and needs no particular observation, the almost colorless appearance of the material in the 90% alcohol being a sufficient guide. This simple technic avoids the necessity for a specially bleached dye solution, the bleaching of material subsequent to staining, the iodine treatment, the clove oil differentiation and consequent long washing in xylol. The method is not intended to take the place of Feulgen staining or the critical fuchsin-light-green technic but is considered to be a distinct improvement on the ordinary gentian-violet-iodine technic.

Crystal violet and gentian violet solutions are easily bleached, of course, by either acid or alkali treatment, just as fuchsin is, but no advantage has been observed from the use of such bleached solutions at present.

It may be of service to give here particulars of the uranium fixative mentioned above: Dissolve 10 g. of chromic acid in 100 cc. of water; then add 20 g. of sodium diuranate. This forms a strong solution which will keep indefinitely. For use, dilute one part with 9 parts of water. Fix for 3 hours or more. Flower buds which are liable to be coated with wax or resinous substances should be dipped for a moment in a mixture of alcohol and chloroform (equal parts) and then transferred to the fixative.

---

[2]Semmens, C. S. 1937. A substitute for osmic acid. The Microscope (London), **1**, 29–31.

Semmens, C. S. 1939. Applications of uranium and certain special fixatives. The Microscope (London), **3**, 3–6.

Bhaduri, P. N. 1940. Advantages of uranium fixation in modern cytological technique. Nature, **146**, 100.

STAINING MYELIN SHEATHS OF OPTIC NERVE FIBERS WITH OSMIUM TETROXIDE VAPOR

RULIN BRUESCH, *Department of Anatomy*[1], *Northwestern University Medical School, Chicago, and Division of Anatomy, University of Tennessee College of Medicine, Memphis*

ABSTRACT.—OsO_4 solution in water, long regarded as the best fixing and staining agent for myelin sheaths, has poor penetrating power. This peculiarity has limited its use to very small pieces of tissue. The vapor from an aqueous solution is known to have a much greater penetrating power for non-neural tissues than the solution itself but nothing has been recorded about its advantages for fixing and staining myelin sheaths of nerve fibers. Difficulties in securing adequate staining of the myelin sheaths in vertebrate optic nerves were overcome largely by the use of the vapor of OsO_4. The technic is carried out as follows: 1) suspend a portion of the nerve above a 2% solution of OsO_4 for 12-24 hours in an air-tight container at room temperature; 2) wash 4–6 hours in distilled water, dehydrate in ethyl alcohol (50% for 2 hours, 70% for 2 hours, and finally 95% overnight), and transfer to *n* butyl alcohol (2 changes of 2 hours each); 3) embed in paraffin, section, mount and cover in balsam in the customary manner.

While there is no doubt that OsO_4 (osmic acid) is the best available fixing and staining agent for myelin, the optic nerve offers more obstacles to successful staining than does a peripheral nerve because it contains numerous fine, closely-packed fibers, without neurilemmal sheaths. Discouraging results with the use of immersion in an aqueous solution of OsO_4 have been recorded by Adrian and Matthews (1927) on the conger eel and by Bishop (1933) on the frog. The author encountered similar difficulties in attempting to obtain satisfactory blackening of the myelin sheaths of fish optic nerves with the usual immersion technics. These experiences led to the development of a vapor technic which has yielded superior results and reduced the number of failures with OsO_4.

HISTORICAL

Brauell (1849) was the first to describe the staining of animal tissues when exposed to OsO_4 in the solid form, in aqueous solution,

[1]Contribution No. 375 from the Department of Anatomy, Northwestern University Medical School.

and as a vapor. His descriptions of the gross appearance of the yellow, brown, or black coloration of tissues after exposure to OsO_4 are detailed, but apparently he did not appreciate that only those containing fat stain black. Schultze (1865) introduced OsO_4 into microtechnic by reporting its use for blackening the end cells of the tracheae of the luminous organs of *Lampyris splendidula*. Schultze and Rudneff (1865) were the first to point out its great value for fixing and staining myelin sheaths. They noted also that OsO_4 penetrates tissues poorly, commenting that the periphery of the spinal cord is well-stained whereas the central region is almost entirely unstained even after many hours of immersion in the solution. Cytologists attempted to overcome this disadvantage of poor penetrability by mixing it with rapid penetrators: combinations of acetic acid, chromic acid, alcohol, $HgCl_2$, bichromate, and platinum chloride, with OsO_4 (Tellyesniczky, 1898). These mixtures still find their principal utility in cytological technics and are of no great value for staining myelin sheaths.

It is often repeated that the vapor of OsO_4 has much greater penetrating power than that of the solution. Just who made the original observation is not clear; Gilson (1885) suggested that the vapors of a freshly prepared mixture of OsO_4 and formic or acetic acid are useful in studying nuclei, and Tellyesniczky (1898) mentioned that the vapor could be used but said nothing about its advantages or disadvantages. Cramer (1919) employed the vapor of a 2% solution at 40°C. for 1½ hours for the demonstration of epinephrine in the cells of the suprarenal gland. Gatenby (1920) pointed out the value of Cramer's procedure and suggested certain modifications of it. No one apparently has published any experiences with the use of vapor for the blackening of myelin sheaths.

Experimental

The comparative penetrating powers of vapor and solution of OsO_4 were put to a trial on the human optic nerve. Two portions of the same nerve, both approximately 0.5 cm. in length and averaging 4 mm. in diameter, were secured; one was immersed in a 2% solution of OsO_4 for 36 hours and the other was suspended above a similar solution in an air-tight container for 24 hours. The experiment was repeated on several different optic nerves, with the following results: the portion of the nerve immersed in the solution showed good demarcation of the peripherally located myelin sheaths, poor differentiation in the middle zones, and feeble staining in the central regions; the portion exposed only to the vapor showed excellent

demarcation of myelin sheaths thruout the entire cross-section of the nerve. The vapor technic was then applied to optic nerves of other vertebrates and successful staining of the myelin sheaths obtained on certain ganoid fishes (*Scaphirhynchus platorynchus, Amia calva*), amphibians (*Bufo americanus, Rana pipiens*), reptiles (*Alligator mississippiensis*), birds (*Gallus domesticus, Columba domestica*), and mammals (*Eptesicus fuscus, Canis familiaris, Felis domesticus, Lepus cuniculus, Mus norvegicus, Mus norvegicus albinus, Cavia cobaya, Sus domesticus, Ovis aries, Macacus rhesus*).

The procedure for the vapor technic is as follows:

1. Suspend the nerve just above the surface of 5 cc. of a 2% aqueous solution of OsO_4 placed in a 50 mm. diameter, low type, Stender dish at room temperature. The nerve is suspended by tying it with silk thread to a glass frame placed in the Stender dish; such a frame can be made simply by bending glass tubing to conform to the size and shape of the dish and allowing the ends to project above the level of the fluid. The duration of fixation in these vapors may be varied from 12 to 24 hours, depending upon the size of the nerve.

2. Wash 4–6 hours in several changes of distilled water.

3. Dehydrate in a graded series of ethyl alcohol, starting with 50% for 2 hours, then 70% for 2 hours, and finally to 95% overnight. Remove the ethyl alcohol by 2 changes of *n* butyl alcohol of 2 hours each.

4. Infiltrate and embed in paraffin.

5. Section 3–5 μ if critical delineation of the myelin sheaths is desired.

6. Mount sections, remove paraffin with xylol, and cover in balsam in the usual manner.

Discussion and Conclusions

Exposure to osmium vapor requires a small air-tight container. The nerve must be suspended just above the level of the solution but never actually touching it. The container must be chemically clean and care must be exercised in introducing the nerve to avoid contaminating the fluid with organic matter. If proper care be exercised, the same solution may be used many times before excessive reduction of the OsO_4 occurs. As soon as the solution becomes blackened, it is unfit for further use. The duration of exposure of the nerve to the vapor is not critical; an interval of 24 hours provides complete fixation and staining for the largest nerves, and much shorter intervals may be used with safety on small nerves.

Washing should be adequate to remove all uncombined OsO_4, for otherwise blackening in alcohol will occur.

Dehydration is completed with 95% ethyl alcohol and the transition to paraffin made with *n* butyl alcohol to avoid too much hardening in absolute ethyl alcohol. The use of *n* butyl alcohol also eliminates the need for immersion of the tissue in xylol.

Two hours at 58°C. is sufficient usually to insure complete infiltration with paraffin. Prolonged periods of exposure to the heat of the oven appreciably harden tissue.

The nerves must be sectioned exactly in cross-section and must be cut as thin as possible (3–5μ) to secure best demarcation of myelin sheaths.

When mounting sections, overheating or prolonged heating with the hot-plate must be avoided because heat produces rapid fading of the blackened myelin sheaths.

Altho this vapor technic was developed for use on the vertebrate optic nerve, its application to any peripheral nerve should yield superior fixing and blackening of myelin sheaths.

LITERATURE CITED

ADRIAN, E. D., and MATTHEWS, R. 1927. The action of light on the eye; discharge of impulses in optic nerve and its relation to electrical changes in the retina. J. Physiol., **63**, 378–414.

BISHOP, G. H. 1933. Fiber groups in the optic nerve. Amer. J. Physiol., **106**, 460–74.

BRAUELL, F. 1849. De acidi osmici in homines et animalia effectu. Comment. physiol. Casani.

CRAMER, W. 1919. Sixth Scientific Report of the Imperial Cancer Research Fund. (Cited from Gatenby, 1920).

GATENBY, J. B. 1920. The cytoplasmic inclusions of the germ-cells. Part VII. The modern technique of cytology. Quart. J. Micr. Sci., **64**, 267–301.

GILSON, G. 1885. La Cellule, **1**, 96 (Cited from Lee, 1928).

SCHULTZE, M. 1865. Zur Kenntniss der Leuchtorgane von *Lampyris splendidula*. Arch. mikr. Anat., **1**, 124–37.

SCHULTZE, M., and RUDNEFF, M. 1865. Weitere Mittheilungen über die Einwirkung der über Osmium Säure auf thierische Gewebe. Arch. mikr. Anat., **1**, 299–304.

TELLYESNICZKY, K. 1898. Über die Fixierungs-(Hartungs)-Flüssigkeit. Arch. mikr. Anat., **52**, 202–47.

A STAINING PROCEDURE FOR USE IN THE BRUCELLA OPSONOCYTOPHAGIC TEST

N. B. McCULLOUGH AND LEO A. DICK, *Brucellosis Research Laboratory, The Clayton Foundation, University of Texas, Austin*

ABSTRACT.—The following staining procedure is recommended for use in the Brucella opsonocytophagic test in order to avoid confusing results obtained with stains of the Hasting or Wright type: Fix spreads for 5 minutes or longer in absolute methyl alcohol. Stain for 10 to 30 minutes in a solution of the following: 0.5 g. NaCl, 0.5 g. phenol, 0.5 g. methylene blue, 0.02 g. $Na_2HPO_4+12H_2O$, 50 cc. distilled water, 50 cc. methyl alcohol. Wash slides gently in water. Air dry. By this procedure, the bacteria and the nuclei of the leucocytes appear deep blue. The cytoplasm of the leucocytes appears faintly green with the cell outline distinctly visible. Cytoplasmic granules do not stain.

Since the introduction of the opsonocytophagic test by Huddleson *et al.*[1] as a quantitative measure of induced immunity in brucellosis of man, it has come into wide use as a diagnostic aid. Huddleson's technic, as well as the many revisions of his test, demands an estimate of the number of bacteria ingested by individual leucocytes. In making this determination it is essential (1) that the bacteria be satisfactorily stained so that they may be clearly seen, (2) that they be easily distinguished from granules of like size occurring in the cytoplasm of the leucocytes, (3) that the leucocytes be preserved from distortion, and (4) that the leucocyte cell membrane be clearly delineated so that it is possible to tell whether marginally situated bacteria are within the cell.

The difficulties encountered in reading such blood spreads are evidenced by the many staining procedures that have appeared in the literature. With the best of these it is frequently impossible to tell whether certain bacteria are within the cell. Either the bacteria are poorly stained or the cytoplasm of the leucocytes is not sufficiently differentiated so that the limits of the cell are easily distinguished. In certain bloods cytoplasmic granules in the leucocytes similar in size and staining properties to the *Brucella* are confusing.

[1]Huddleson, I. F., Johnson, H. W., and Hamann, E. E. 1933. Study of opsonocytophagic power of blood and allergic skin reactions in *Brucella* infection and immunity in man. Amer. J. Pub. Health, **23**, 917–29.

This is particularly true when methods employing the common blood stains are used.[2]

When the opsonocytophagic technic is applied to the blood of animals other than the human[3] the results are even more confusing. The published staining procedures have proven worthless for this test with the blood of the ox, rabbit and guinea pig. The leucocytes of all these species are rich in cytoplasmic granules.

The staining procedure to follow has been devised to obviate some of the difficulties encountered in the use of the various published procedures.

Spreads are prepared as usual. The authors prefer to use Huddleson's technic for making these spreads. It should be emphasized that this is the most important single step in the preparation of satisfactory slides. One should avoid thick spreads. If made by the draw method, the spreads should not be allowed to terminate abruptly as this results in crowding of the leucocytes. The spreads should be dried rapidly to prevent distortion of the cells.

Stain: Fix the spreads for 5 minutes or longer in absolute methyl alcohol (acetone free). Stain for 10 to 30 minutes in a solution of the following:

0.5 g. NaCl
0.5 g. phenol
0.5 g. methylene blue (certified)
0.02 g. $Na_2HPO_4+12H_2O$
50 cc. distilled water
50 cc. methyl alcohol (absolute)

After staining wash the slides gently in tap water. Do not prolong washing. Allow spreads to dry in air.

The fixing and staining are done in Coplin jars. Spreads may be left in the fixing jar for several hours without harm. The optimum staining period varies with the blood of different species, with the thickness of the spreads, with the time interval between making and staining the spreads, and possibly with other factors. The staining solution may be used until depleted of dye.

[2]Huddleson recommends the use of Hasting Stain prepared by a particular manufacturer. We have compared the staining properties of this particular Hasting Stain with those of Hasting Stain prepared by two other manufacturers. Since Hasting and Wright Stain are usually identical preparations bearing different labels, we included in this comparison seven samples of Wright Stain obtained from four different manufacturers. As regards the test in question there was no essential difference in the staining properties of these ten samples.

[3]Huddleson, I. F., Johnson, H. W., and Meyer, D. B. 1936. A method for measuring the opsonocytophagic power of the blood of cattle for *Brucella*. Technical Bulletin No. 149, Michigan Agricultural Experiment Station.

In the stained preparation the bacteria and the nuclei of the leucocytes appear deep blue. The cytoplasm of the leucocytes appears faintly green with the cell outline distinctly visible. The intensity of the cytoplasmic staining may be varied to suit the individual by shortening or lengthening the staining period. Acidophilic granules fail to stain. The smaller normal basophilic and neutrophilic granules either remain unstained or take a light green stain blending with the stained cytoplasm. The large granules in basophilic leucocytes stain light purple and are easily distinguished from bacteria by their staining reaction and their size. The erythrocytes are preserved and have a greenish-blue appearance in the stained spread.

This staining procedure has proven satisfactory for use with the blood of the human, ox, sheep, rabbit and guinea pig.

NOTES ON MOUNTING MEDIA

J. L. Mohr and Wm. Wehrle[1], *University of California, Berkeley, Calif.*

A. Media in Place of Green Euparal of Gilson.

Among the materials of the microscopist which may become unobtainable at any time are certain proprietary mountants. It would be desirable at any time to replace such media (caedax, euparal, Gurr's neutral mounting medium, etc.) because they are of composition uncertain to the average user. Therefore their behavior is not very predictable and they cannot be manipulated well as to viscosity, refractive index, etc. They are typically rather high in price and in time of war may be unpurchaseable.

One proprietary medium widely used is green euparal of Gilson[2]. This is composed of unpublished proportions of camphor, salol, eucalyptol, sandarac and paraldehyde with a copper salt for coloring. Because of its relatively low refractive index (1.483), it is a good medium for demonstration of cytological detail, especially where alternate slides may be studied in clarite or another medium of appreciably higher refractive index. Further virtues are ease of use (materials may be mounted from 96% alcohol) and suitability for use with Romanowsky stains. On the other hand, some workers report[3] that hematoxylin stains deteriorate in euparal. This has not been the experience of the writers who have used proprietary euparal only four years.

Whether or not euparal is unsuitable for permanent mounts of materials stained in hematoxylin, a mountant of this general character has many proper uses. For that reason the compounding of two related media is described. Manufacture is not difficult if the sequence given is followed. The media may be altered by changing proportions or substituting related ingredients to raise or lower viscosity, refractive index, etc. The amounts given are for "institutional" quantities, but smaller amounts are handled somewhat more easily.

---

[1]Formerly employed by the Works Progress Administration. Aid from the United States Works Projects Administration (Official Project 265-1-08-80, Unit C 1) is hereby acknowledged.

[2]Gilson, G. 1906. Un nouveau médium solidifiable pour le montage des préparations microscopiques. La Cellule, 23, 427.

[3]See Gatenby, J. B. 1937. Lee's Microtomist's Vade-mecum. *10th Ed.*, p. 227–8.

For more than three years the following mounting media, compounded by the junior author, have been used in the Department of Zoölogy at Berkeley. Equal parts of camphor (synthetic of Dupont) and salol C.P. or U.S.P. (phenyl salicylate) are granulated and mixed. In a day or two there results a clear, water-white saturated solution with 2–3% residue of camphor. This is Gilson's "camsal". To 100 g. of camsal is added 400 g. of selected tears of gum sandarac (Eimer and Amend or Penick). Two hundred grams of eucalyptol U.S.P. (Merck, Mallinckrodt, or any good grade), 200 g. of dioxan C.P., and 100 g. of paraldehyde U.S.P. are mixed with the 500 g. of camsal and sandarac in a large bottle and the bottle is shaken from time to time until solution is complete.

The solution must be filtered thru coarse filter paper while it is covered to minimize evaporation. In four or five days there remains a viscous residue of about 850 g. This may be diluted to 1000 g. with dioxan if a thinner mounting medium is desired. To finish the product a solution of 1 g. copper oleate in 9 cc. eucalyptol is added, drop by drop, as needed to color to pale green (not all will be needed).

As Gilson noted in the case of green euparal, this green dioxan medium may turn to a dark yellow in the absence of light. In the sunlight the green color returns. The mountant should be stored in a bottle not more than ⅔–¾ full and should be kept in sunlight if convenient. A batch of the mounting medium compounded three years ago shows no signs of deterioration. The refractive indices of samples from two batches were 1.493 and 1.496.

Sections or smeared material may be mounted in the medium from the higher alcohols or from dioxan. It is not offered for permanent mounts of materials prepared with any dioxan-soluble stain because materials so stained will lose color. This fact seriously limits its use. In the zoölogical laboratories here, however, numbers of exploratory stains on nitrocellulose and paraffin sections and study smears of *Drosophila* salivary gland chromosomes are so mounted for convenience and economy. *Microcystis* (blue-green alga) stained in weak alcoholic fast green FCF and dinoflagellates (peridinians) stained in Lynch's precipitated borax-carmine or iron hematoxylin have been mounted in this medium satisfactorily after a brief washing in clean 96% alcohol.

A mountant which goes into solution more slowly is made as follows: Wrap 300 g. clean tears of gum sandarac in a brass screen. Suspend this in a solution of 600 g. eucalyptol and 75 g. camsal compounded as described above. Keep in a tightly closed container in a moderately warm place (as on top a paraffin oven) and open to stir

several times daily until the gum is entirely dissolved. Finally add 25 g. of paraldehyde (which is not a solvent for gum sandarac), add eucalyptol to the liter mark, and color with a very small amount of the copper oleate in eucalyptol solution described above. Mix well before filtering thru very coarse filter paper in a closed vessel. This medium is probably quite close in composition to Gilson's original green euparal.

B. Plasticizer with Clarite.

Since Groat[4] suggested the use of clarite, first sold under the name Nevillite V, in 1939, it has enjoyed increasing popularity (as well as a material advance in price). The popularity has come about, in the opinion of the writers, primarily from favorable publicity and from its pleasing water-whiteness. The excellent color of hematoxylin preparations made in 1939 and early in 1940 and mounted in clarite-toluene solutions corroborates the contention of Groat that the chemical inertness of clarite is advantageous. With his statement that bubbles are easily eliminated in the clarite-toluene solutions in the proportions he recommends, we can find no agreement. In smear preparations bubbles caught are not always self-eliminating and they are not easily eliminated. Rather more serious are the bays of air which frequently replace toluene evaporated from deep mounts. Further the medium is very intolerant to traces of water.

Because clarite deep mounts are clearer than either Canada balsam or damar mounts, we suggest two methods for avoiding air-bays. The first is to infiltrate objects slowly with clarite in toluene until the consistency equals that of a 60% clarite solution. At this point the object is transferred to a microscope slide with a glass cell of suitable depth. The cell is filled to the brim with clarite solution and the slide is placed under a bell jar, to exclude dust, while toluene evaporates. After a week the cell is again filled and several days later fresh clarite added and a cover slip is applied.

A second method is to employ up to 10% of the plasticizer, dibutyl phthalate, in the clarite solutions. Such solutions are much less subject to air-bay trouble than the regular clarite solutions. In a small test series of thirty slides of smears of anuran rectal contents, made more than a year ago, 15 without plasticizer and 15 with, three without plasticizer have developed air-bays while all with dibutyl phthalate are unmarred. The hematoxylin stain remains unfaded in both parts of the series. Plasticized clarite mounts dry more

[4]Groat, R. A. Two new mounting media superior to Canada balsam and gum damar. Anat. Rec., 74, 1. 1939.

slowly, however, and in deep mounts have remained fluid more than four months. Accordingly, we recommend the first method for whole mounts of large flukes, annellid worms, chicks, etc. and the plasticizer-solutions for sections, smears, and small whole mounts.

STAINING ASPIRATED HUMAN BONE MARROW WITH DOMESTIC WRIGHT STAIN

EMIL MARO SCHLEICHER, *Hematological Laboratory, Institute of Anatomy, University of Minnesota, Minneapolis*

ABSTRACT.—The method employs the domestic Wright stain for the staining of aspirated human bone marrow. Freshly distilled water, pH 6.0 to 6.4, is used. Wright stain, 0.5 cc., is placed upon the air-dried preparation and permitted to act for two minutes. The stain is then diluted with 2 cc. distilled water and permitted to act for from 5 to 10 minutes. After washing off the stain with distilled water, the preparation is placed into a decolorizer (acetone 0.5 cc., pure methyl alcohol 5.0 cc., and 100 cc. distilled water, pH 6.0 to 6.4) for differentiation from 1 to 5 seconds, rinsed, washed under running water and permitted to air-dry. A well stained and differentiated preparation shows the "Romanovsky effect", and the sharpness of minute structures obtained compares favorably with control preparations stained with German dyes.

The bone marrow should be prepared as described. The Wright stain marketed by the National Aniline and Chemical Co., N. Y. was found to be reliable as regards staining quality of registered batches. One photomicrograph, showing bone marrow cells from pernicious anemia, is included.

German compound blood stains have been used extensively for aspirated human bone marrow for research purposes. There are two reasons for this preference of German dyes over the domestic products: (a) uniformity and reliability of successive batches, and (b) the clear-cut differentiation of minute morphologic details. The lack of these features of domestic Wright stain led to the employment of buffered diluents ranging from pH 6.0 to 7.0. This variation of H-ion concentrations represents in final analysis individual judgment as regards optimum staining quality rather than adjustment of the buffered diluent to the individual dye batches. A large number of general clinical laboratories use distilled water. Samples obtained from various institutions have ranged in H-ion concentration from 5.35 to 6.42 with 68.4% of the samples testing about pH 6.0.[1] Examination of marrow preparations stained with domestic Wright stain, using as diluent distilled water ranging from

[1]The determinations were made with a pH electrometer.

pH 5.35 to 6.42, revealed that distilled water with an H-ion concentration of from 6.0 to 6.42 is suitable for Wright stain. The preparations failed to show the "Romanovsky effect[2]", however, as has been claimed by some users of distilled water. The staining variability of individual dye batches is the chief problem at hand, and while distilled water does not correct this undesirable feature, overstaining generally occurs when buffered diluents above pH 6.4 are used. Thus minute morphologic structures, especially in immature cells, are not sharply defined, and proper identification of the elements is, therefore, precarious. Experiments showed that regressive staining

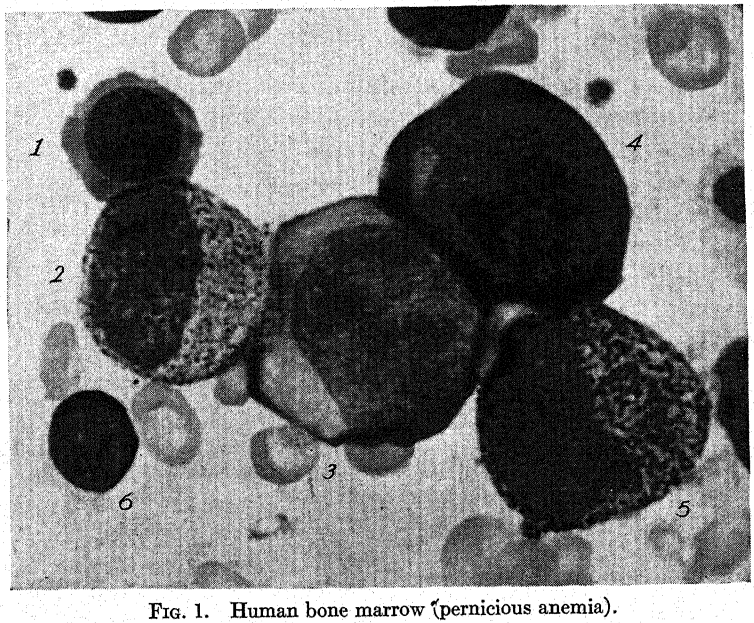


FIG. 1. Human bone marrow (pernicious anemia).

1) Polychromatic normoblast. 2) Promyelocyte. 3) Promegaloblast. 4) Basophilic megaloblast. 5) Leukoblast, late stage. 6) Lymphocyte.

was the solution to the problem not only to obtain sharply defined minute structures but also to produce the much desired "Romanovsky effect". The latter phenomenon is obtained by exposing the stained preparation to a decolorizer. Preparations treated with the method given below compare favorably with those stained with German compound dyes.

It is not within the scope of this paper to enter into a detailed

[2]Romanovsky effect: color of nuclei red lavender, nucleoli deep to light blue, and cytoplasmic granules of all types well differentiated.

analysis of the German and domestic compound stains; the reader is referred to the splendid chapter on "Compound Dyes" in H. J. Conn's Biological Stains[3].

Method for preparing the bone marrow. The method of aspirating bone marrow has been described by the writer elsewhere[4]. After the marrow has been obtained it is placed in a paraffin-lined small test tube containing a small amount of powdered heparin and mixed gently. A Wintrobe hematocrit tube is filled with this mixture and centrifuged at about 1500 to 2000 RPM for 3 to 5 minutes. Centrifugation separates the marrow into the following layers from top of the tube down (a) fat, (b) plasma, (c) myeloid-erythroid, and (d) mature erythrocytes (sinusoidal blood). The divisions occupied by the myeloid-erythroid (M-E) layer are noted and an equal number of divisions of the plasma layer marked off. The fat and the remaining supernatant plasma are drawn off. The marked-off plasma and the M-E layers are now removed and gently mixed in a paraffin-lined watch glass and a thin margin-free smear made and stained as soon as the marrow has air-dried.

Material:

I. Wright stain (dry powder) certified, (National Aniline)..... 0.1 gram
Special pure methyl alcohol, (National Aniline).. 60.0 cc.

(Rub up the powder with the alcohol in an absolutely clean, dry mortar. Permit the stain to stand for 36 hours in a suitable container and shake frequently. Filter the stain and store in a dark glass bottle.)

II. Decolorizer
Acetone..... 0.5 cc.
Methyl alcohol..... 5.0 cc.
Freshly distilled water, pH 6 to 6.4.....100.0 cc.

(Do not use the decolorizer immediately after mixing since the unstable thermal state offsets the even action of the decolorizer.)

III. Freshly distilled water pH 6.0 to 6.4

Method of staining:

1. Cover the preparation with 0.5 cc. of the Wright stain and permit to act for two minutes.
2. Add 2 cc. of distilled water, mix well and stain for from 5 to

[3]Conn, H. J. 1940. Biological Stains. *4th Ed.* Biotech Publications, Geneva, N. Y.
[4]Schleicher, E. M., and Sharp, E. A. 1937. Rapid methods of preparing and staining bone marrow. J. Lab. and Clin. Med., 22, 949.

10 minutes. Wash the stain off with distilled water. (While still wet, check the affinity of the stain under the microscope, i.e. its selective action upon certain cells or cell structures.)

3. Leave the preparation in the decolorizer for 1 to 5 seconds. Dip preparation in distilled water for a few seconds, keeping the slide in slight motion. Rinse under running distilled water to assure complete removal of excess stain and decolorizer.

(Keep in mind that the individual Wright stain determines the exposure time in the decolorizer and the amount of washing to be done to obtain extreme precision of the differentiation of the cells to be studied. Careful staining experiments showed that blood stains are not "panoptic" in the true meaning of the word.)

4. Permit the preparation to air-dry.

Fig. 1, a photomicrograph of bone marrow cells from pernicious anemia stained with the method described above illustrates the clearness and sharpness of the morphologic details which can be brought out by controlling regressive staining under the microscope. The cell in the left upper corner is a polychromatic normoblast and directly below it is a promyelocyte with a few azure and a small quota of neutrophilic granules. The cell in the center is a promegaloblast with a "myeloblast-like" nucleus, and above this is a basophilic megaloblast derived directly from the reticulum. In the lower right corner is a late leukoblast with large dark-staining azure granules and among them a small quota of neutrophilic granules. A lymphocyte is seen in the left lower corner.

Peripheral blood may be stained the same way as the bone marrow.

Wright stain samples (dry powder) bearing certification numbers NWr-4, 10, 11, 14–17, and marketed by the National Aniline and Chemical Co. N. Y. were used for dye analytical studies. All dye batches yielded satisfactory preparations.

Acknowledgment. I wish to express my sincere thanks to Dr. Hal Downey who was kind enough to examine many preparations for morphologic details. The photomicrograph was made by Mr. H. W. Morris, campus photographer.

THE USE OF THE BENZIDINE METHOD ON THICK SPECIMENS

ROBERT A. PFAFF AND W. LANE WILLIAMS, *Department of Anatomy, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.*

ABSTRACT.—**A method, employing benzidine, nitroferricyanide and hydrogen peroxide, for demonstrating blood vessels, was applied to formalin-fixed specimens of whole mouse organs and to rat embryos ranging in thickness up to 5 mm. This staining technic outlined the blood vessels by reacting with the hemoglobin of intraluminal erythrocytes. The specimens were treated in a mixture of 5% benzidine in 2.5% acetic acid, and 5% aqueous sodium nitroferricyanide; then in dilute hydrogen peroxide (4–6 drops H_2O_2 to 100 cc. water). After dehydrating in alcohol and dioxan, clearing in cedar oil or xylol, they were mounted in balsam or stored in cedar oil. The staining procedures were carried out at 37° C.**

In recent years several staining methods have been devised to demonstrate vascular channels. Many of these technics have been based upon the increased degree of selective staining affinity possessed by erythrocytes for certain dyes, or upon the ability of the red blood cells to retain the coloring agents in the presence of destaining or differentiating substances. Among these are the benzidine method (Pickworth, 1934; Campbell, Alexander and Putnam, 1938; Doherty, Suh and Alexander, 1938), the chromotrope 2R method (Crossmon, 1940), the method using iron alum, acid hematoxylin, and lithium carbonate (Smith and Quigley, 1937), and the acid fuchsin method (Eros, 1941; Eros and Priesman, 1942). The first of these is the most generally used at present.

Benzidine (p-diamino-diphenyl) has long been used in tests for the detection of occult blood and is a component of several peroxidase stains for blood smears (Mallory, 1938; Stitt, Clough and Clough, 1938). Lepehne (1919) used benzidine to demonstrate blood pigments in macrophages and parenchymal cells during icteric conditions. More recently, solutions of benzidine, nitroferricyanide, and hydrogen peroxide have been utilized for the histological demonstration of smaller blood vessels. Such vessels are well outlined as a result of the reaction of these reagents with the hemoglobin of intraluminal erythrocytes. For the most part, use of the benzidine method has

been limited to formalin-fixed tissue of the central nervous system sectioned at 200–300 μ on a freezing microtome (Pickworth, 1934; Campbell *et al.*, 1938; Doherty *et al.*, 1938; Bergmann, 1942). Such procedures give excellent demonstrations of the vascular pattern of the central nervous system and are of great aid in studying certain lesions of the same. The technic has also been applied to fairly thin frozen sections of the guinea pig uterus in studying cyclic alterations in its vascular architecture (Bacsich and Wyburn, 1940). The purpose of the present study was to determine whether or not the benzidine method which has previously been applied to fairly thin frozen sections could be successfully used in the demonstration of the vascular channels present in small whole organs.

Materials and Methods

The following tissues were treated by the method using benzidine, nitroferricyanide, and hydrogen peroxide: reproductive tract, mammary glands, small intestine, and the ventral abdominal musculature of a multiparous albino mouse; 16-day fetuses (whole and sagittally halved) and placentae from normal and vitamin-E-deficient rats. The mammary glands of the mouse were 2–3 mm. in thickness and the uterus was 5 days post parturient, thick, and edematous. The whole specimens and portions of rat fetuses varied from 3–6 mm. in thickness. In some instances whole placentae were treated; in others, frozen sections cut at 200 μ were used.

Fixation: All tissues were fixed for at least 48 hours in 10% formalin. The mammary glands were fixed on the skin which was pinned out on a cork board. After fixation the glands were dissected from the skin. The ventral abdominal musculature, reproductive tract and intestine (cut along its longitudinal axis) were also fixed in a similar manner on cork boards. The embryos were fixed *in toto*, and when such were to be cut in half, the specimens were frozen and then sectioned sagittally with a razor blade.

Solutions: The staining solutions were made up according to the formulae of Campbell, Alexander and Putnam (1938).

Solution A

Benzidine base, c.p. (National Aniline)	0.1 g.
Glacial acetic acid	0.5 cc.
Distilled water	20.0 cc.

Solution B

Sodium nitroferricyanide	0.1 g.
Distilled water	20.0 cc.

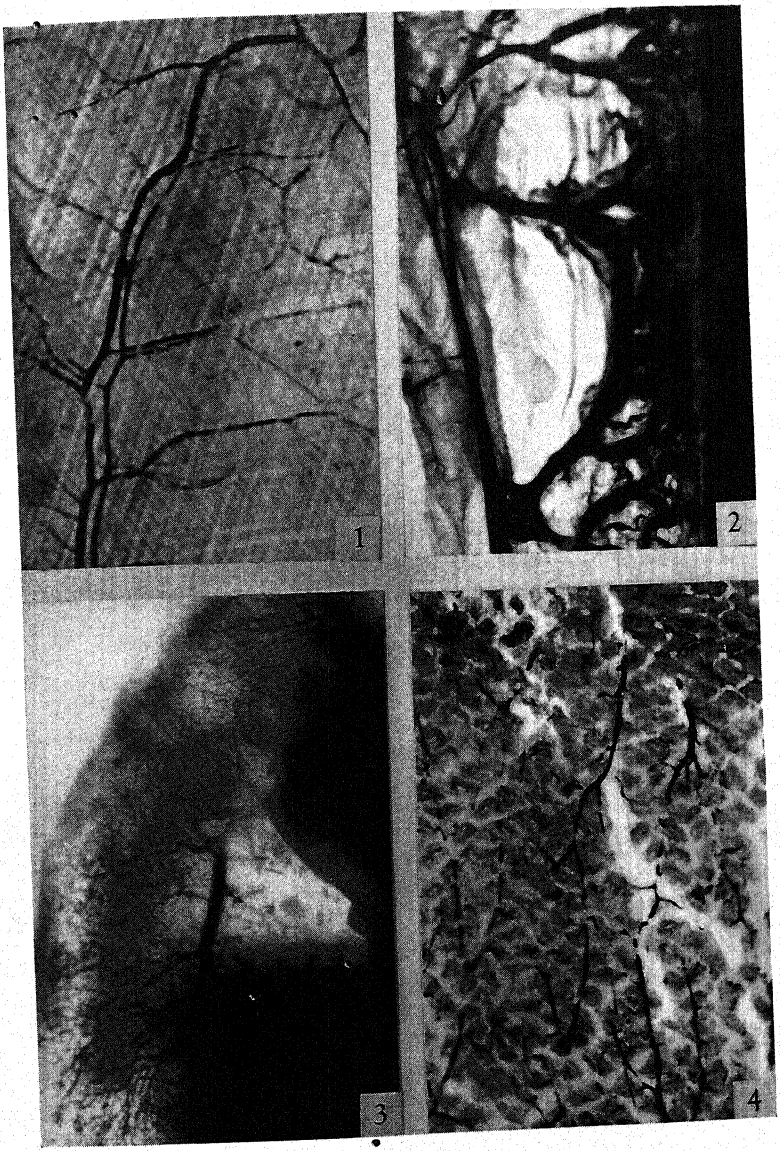


PLATE 1. Explanation of Figures.

1. Vessels in ventral abdominal musculature (mouse). ×10.
2. Uterine vessels (mouse). ×10.
3. Portion of a 16-day rat fetus showing vessels in the body wall from the outside in an area on the right side, extending from the shoulders (upper dense area) to slightly below the level of the liver (lower dense area). Fetus was from a vitamin-E-deficient mother and there is some vascular stasis and dilation of superficial vessels. ×20.
4. Vessels of small intestine (mouse). The mucosal surface of a flattened and stretched piece of intestine is shown. ×20.

Solution C

30% hydrogen peroxide (Superoxol) ..... 4 to 6 drops
Distilled water ..... 100 cc.

All of the solutions were made up fresh just before using.

Staining Procedure:

(1) Immediately prior to use, combine solutions A and B and make up to 100 cc. with warm (37° C.) distilled water.

(2) Place specimens to be stained in this combined solution for 30–45 min. at 37° C. and stir several times during this period.

(3) Remove specimens from staining solution and wash in a fairly large volume (e.g. 500 cc.) warm distilled water (37° C.) for several minutes.

(4) Put tissues in solution C for ½ hour at 37° C. The vascular areas assume a blue color during this time.

(5) Wash in about 500 cc. of warm water (37° C.).

(6) Dehydrate by the use of alcohols or dioxan, or both.

(7) Clear in xylol or cedar oil, storing in the latter if necessary.

(8) Mount in balsam under glass (or store in cedar oil).

Remarks

The complete procedure should be carried out in clean glassware. Metal "carrying" trays and stirring instruments should not be introduced into the solutions. Certain samples of technical "benzidine" were not satisfactory, particularly as to solubility in solution A. The use of cold water for washing caused puckering and folding of some of the thicker specimens.

The photographs (Fig. 1–4) show the appearance of the blood vessels at magnifications of 10× and 20×. Examination of the preparations at higher magnifications revealed that the clarity of the outlines of blood vessels was due to the selective staining of the erythrocytes, in colors ranging from dark brown to black after clearing, and not from the staining of mural elements of the vascular channels. In the uterus and vagina both the intramural as well as extramural vascular patterns were well demonstrated, altho the former do not show clearly in the photograph (Fig. 2). The vessels of the mammary glands were also clearly outlined despite the thickness of the glands and their considerable fat content. In the fetuses the relatively large muscular masses of the extremities and also the livers appeared dark and dense (Fig. 3). The same was true for the muscular wall of the uterus. The fetus in Fig. 3 is from a vitamin-E-deficient rat and the superficial vessels show the stasis and dilation which often occur in this avitaminosis (Mason, 1942 a and b).

Specimens which had been stored in cedar oil for 4 months were still intact as to the staining of their vascular architecture. Such material is most satisfactory for study and demonstration with a dissecting microscope.

The size of the specimens put the method to a fairly critical test. Because of the thickness of the tissues the solutions did not penetrate uniformly to all of the vessels. However, the results showed that the benzidine method which had been formerly applied chiefly to frozen sections 100–300 μ in thickness could also be used to demonstrate the general vascular pattern of certain whole organs and other relatively thick specimens.

REFERENCES

BACSICH, P., and WYBURN, G. M. 1940. Cyclic variations in the vascular architecture of the guinea pig uterus. Trans. Roy. Soc. Edinburgh (Part I), **60**, 79–86.

BERGMANN, L. 1942. Studies on the blood vessels of the human Gasserian ganglion. Anat. Rec., **82**, 609–30.

CAMPBELL, A. C., ALEXANDER, L., and PUTNAM, T. J. 1938. Vascular pattern in various lesions of the human central nervous system. Arch. Neurol. and Psychiat., **39**, 1150–202.

CROSSMON, G. 1940. The selective staining of red blood cells. Stain Techn., **15**, 155–8.

DOHERTY, M. M., SUH, T. H., and ALEXANDER, L. 1938. New modifications of the benzidine stain for the study of the vascular pattern of the central nervous system. Arch. Neurol. and Psychiat., **40**, 158–62.

EROS, G. 1941. Method for fuchsin staining of the network of cerebral blood vessels. Arch. Path., **31**, 215–9.

EROS, G., and PRIESTMAN, G. 1942. Cerebral vascular changes in carbon monoxide poisoning (report of a case). J. Neuropath. and Exp. Neurol., **1**, 158–72.

LEPEHNE, G. 1919. Zerfall der roten Blutkörperchen beim Ikterus infectiosus (Weil). Beitr. Path. Anat. u. Allg. Path., **65**, 163–226.

MALLORY, F. B. 1938. Pathological Technique. W. B. Saunders Co., Philadelphia.

MASON, K. E. 1942a. A hemorrhagic state in the vitamin E deficient fetus of the rat. Univ. Cal. Pub. Anat. (in press).

———— 1942b. Changing concepts of the antisterility vitamin (vitamin E). Yale J. Biol. and Med., **14**, 605–17.

PICKWORTH, F. A. 1934. A new method of study of the brain capillaries and its application to the regional localisation of mental disorder. J. Anat., **69**, 62–71.

SMITH, W. K., and QUIGLEY, B. 1937. A new method for the rapid staining of myelin sheaths. Amer. J. Path., **13**, 491–5.

STITT, E. R., CLOUGH, P. W., and CLOUGH, M. C. 1938. Practical Bacteriology, Haematology and Animal Parasitology, *9th Ed.* The Blakiston Co., Philadelphia.

CYTOLOGICAL METHODS FOR CREPIS SPECIES

H. A. TOBGY, *Division of Genetics, University of California, Berkeley, California*

ABSTRACT.—Root tips of Crepis species are fixed in La Cour's "2BE" and dehydrated thru a butyl alcohol series. They are stained in 1% crystal violet for 1 hour, with chromic acid and iodine as pre- and post-staining mordants, respectively, and passed thru dehydrating alcohols containing picric acid and ammonium hydroxide. Differentiation is done in clove oil. The method is rapid; the chromosomes are dark purple; the centromere is not stained; and the cytoplasm is clear. By further controlled destaining the heterochromatic segments within the chromosomes may be located.

Pollen mother cells are fixed in acetic alcohol (1:4) and squashed in aceto-carmine. A method is described for making semi-permanent preparations mounted in diaphane.

Pollen grains are mounted in lacto-phenol with acid fuchsin or anilin blue W. S. as the dye.

The genus *Crepis*, which belongs to the tribe Cichorieae of the family Compositae, comprises some 195 species; of these 113 have, so far, been examined cytologically (cf. Babcock, 1942). Most of these species are a favorable material for chromosomal studies, but some of them are rather difficult to stain satisfactorily. The cytological technics used with the *Crepis* species have been steadily improved and it is proposed to outline here the methods used during a recent investigation at the Division of Genetics of the University of California.

1. ROOT TIPS

Fixatives of the Navashin type (chromic acid, formalin, and acetic acid) are quite satisfactory for general chromosome surveys. Their characteristic effect of slightly swelling and shortening the chromosomes is not undesirable in general routine work, and, indeed, it may facilitate chromosome counting. They, however, have one serious disadvantage: the centromere is often completely stained or at least more or less obscured. The exact location of the centromere is important for identifying the individual chromosomes and even more so for making accurate length measurements of the chromosome arms. For these two purposes fixatives of the Flemming type (chromic acid, osmic acid, and acetic acid) are unexcelled since they allow the

centromere to remain unstained. In a trial involving four such fixatives: Flemming-strong, Flemming-Benda, Champy, and La Cour's "2BE", (1937) all have given the same fine results; and "2BE" is thus recommended because it is the least expensive. The schedule for fixation and dehydration follows:

1. Fix in "2BE" for 20–24 hours.
2. Wash in tepid water (30°C.) on top of the paraffin oven, changing the water half-hourly over a period of four hours.
3. Pass thru 25% and 50% ethyl alcohol, each for two hours.
4. Complete dehydration and infiltration using Randolph's *n*-butyl alcohol series (1935). Imbed in paraffin. Changing the alcohols is facilitated by placing the material in perforated brass baskets and using a Buchner funnel as described by Duffield (1940).

For staining root tips, Stockwell's modification (1934) of the triple stain is the standard method for *Crepis* species in this laboratory. It usually gives good results, altho in the case of a few species, such as *Crepis neglecta* and *C. fuliginosa* the results are not quite as satisfactory. With these two species there is always an excess of safranin, left in the cytoplasm, which is difficult to remove without destaining the chromosomes themselves. A modified schedule has been tried for these and for similarly difficult species. The safranin and orange G are eliminated from Stockwell's procedure and the crystal violet is used alone. The method differs, however, from Newton's original gentian-violet-iodine stain (1926) in several points, mainly in the application of a 1% aqueous chromic acid as a pre-staining mordant and the differentiation in a saturated picric acid alcoholic solution as first suggested by Johansen (1932) and Smith (1934), followed by a treatment with NH_4OH as given by Stockwell (1934). The results obtained by this method are superior in every respect. The chromosomes are stained a dark and intense purple; the disagreeable transparency of the original gentian violet method is eliminated; the centromere is left as a stainless gap; the cytoplasm is faintly stained in yellow; and the difficulties of an excess of safranin are no longer present. The modified schedule is as follows:

1. Remove paraffin and run slides down to 70% alcohol.
2. Bleach in equal parts of 95% alcohol and commercial hydrogen peroxide (3% H_2O_2) for half an hour.
3. Wash briefly in water and then mordant in a 1% aqueous solution of chromic acid for half an hour.
4. Wash briefly in water and then stain for 1 hour in a 1% aqueous solution of crystal violet.

5. Wash briefly in water and then mordant for 30 seconds in a solution of 1 g. iodine plus 1 g. KI in 100 cc. of 50% alcohol.
6. Pass thru this alcohol series for a few seconds each: 70% alcohol; 95% alcohol saturated with picric acid; 95% alcohol to which a few drops of NH_4OH have been added; 95% alcohol; 100% alcohol.
7. Differentiate in clove oil under the microscope.

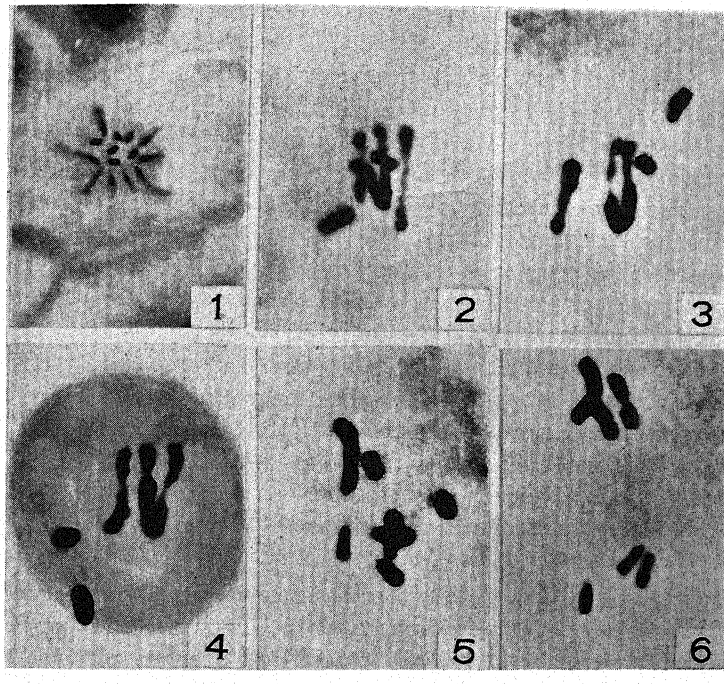


FIG. 1. A root-tip cell in *Crepis neglecta* (2n=8). Note the heterochromatic segments within the chromosomes. Crystal violet.

FIG. 2–6. Pollen mother cells of the hybrid *C. neglecta* (n=4)×*C. fuliginosa* (n=3). Aceto-carmine.

(All photomicrographs are at ×1500.)

8. Wash thoroly in xylol; 3 jars for about half an hour each.
9. Mount in balsam.

It is also possible, by using the above method and by carefully destaining the chromosomes in the clove oil, to differentiate the heterochromatic segments within the somatic chromosomes (Fig. 1).

For making aceto-carmine root-tip smears the method of Warmke (1935) and its modification by Brown (1937) are successful with the

Crepis species. These smear preparations are used for rapid determinations of chromosomes numbers but they cannot be used for making comparative chromosome measurements since the identity of the tissue layers to which a given cell belonged can no longer be recognized. Paraffin-embedded material should be used for making such measurements.

2. Pollen Mother Cells

Several workers have used paraffin material for studying the meiotic divisions in *Crepis*. Preparations made from such material and stained with iron hematoxylin or crystal violet, tho permanent, are not as favorable for making detailed chromosome studies as those fixed in acetic alcohol and smeared in aceto-carmine. Altho their keeping qualities are bad, aceto-carmine preparations are indispensable for an accurate analysis of chiasma conditions and complex chromosome configurations.

The floral buds are fixed in a mixture of 1:4 glacial acetic acid and absolute alcohol for 24 hours, washed thoroly, and stored in 70% alcohol. Smears are made by squashing 3 or 4 florets on each slide in a drop of aceto-carmine, with or without iron, as prepared by Belling (1926). Special care should be taken in heating and pressing the preparations and in removing most of the anther debris so as to get well flattened pollen mother cells (Fig. 2–6); detailed studies of meiosis can only be made on such cells.

These temporary preparations are made permanent according to the following method:

1. Float off the cover slips in 1:1 acetic acid and absolute alcohol.
2. Wash rapidly, using a dropper, with: 1:3 acetic acid and absolute alcohol; then 1:9 acetic acid and absolute alcohol; and finally absolute alcohol.
3. Mount in diaphane.

Such preparations keep fairly well for a period not exceeding six months.

3. Pollen Grains

Pollen grains are mounted in lacto-phenol in which either acid fuchsin or anilin blue W.S. is dissolved. The following formula, which is one of several formulae given by Maneval (1936), is used:

Phenol	20 cc.
Lactic acid	20 cc.
Glycerin	40 cc.
Water	20 cc.
1% aqueous acid fuchsin (or anilin blue W.S.)	1–5 cc.

This mounting medium is more convenient than aceto-carmine. No sealing is required and the mounts keep for months without deterioration.

The writer is indebted to Dr. J. A. Jenkins and to Mr. Ernest Jund, Division of Genetics, University of California, for their kind and continuous help.

REFERENCES

BABCOCK, E. B. 1942. Systematics, cytogenetics and evolution in *Crepis*. Bot. Rev., **8**, 139–90.

BELLING, J. 1926. The iron-aceto-carmine method of fixing and staining chromosomes. Biol. Bull., **50**, 160–2.

BROWN, W. L. 1937. A modified root-tip smear technic. Stain Techn., **12**, 137–8.

DUFFIELD, J. W. 1940. Time savers for fixing and dehydration. Stain Techn., **15**, 57–60.

JOHANSEN, D. A. 1932. A new method for differentiating gentian violet when used as a somatic chromosome stain. Stain Techn., **7**, 17–20.

LA COUR, L. 1937. Improvements in plant cytological technique. Bot. Rev., **3**, 241–58.

MANEVAL, W. E. 1936. Lacto-phenol preparations. Stain Techn., **11**, 9–12.

NEWTON, W. C. F. 1926. Chromosome studies in *Tulipa* and some related genera. J. Linn. Soc., **47**, 339–54.

RANDOLPH, L. F. 1935. A new fixing fluid and a revised schedule for the paraffin method in plant cytology. Stain Techn., **10**, 95–6.

SMITH, F. H. 1934. The use of picric acid with the Gram stain in plant cytology. Stain Techn., **9**, 95–6.

STOCKWELL, P. 1934. A stain for difficult plant material. Science, **80**, 121–2.

WARMKE, H. E. 1935. A permanent root-tip smear method. Stain Techn., **10**, 101–4.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

GRAHAM, CLARENCE F. **Simplification of fluorescent microscopy.** *J. Lab. & Clin. Med.*, 27, 1189–91. 1942.

Instead of a special light in a dark room, a powerful source of light (like a carbon arc, or a 500 watt projection bulb fitted with a condensing lens of about 7 cm. focal length with a blue filter in front of the substage condenser) may be used in a room darkened only by drawing the shades. A 500 watt G.E. projection bulb with a pair of ten-cent-store reading glasses mounted in an improvised sheet metal lamp box is satisfactory. The reading glasses are arranged in tandem, the proper relationships being determined by trial and error. An ordinary mirror is as good as the usual aluminum mirror. Methyl salicylate is the best immersion oil for this purpose.—*John T. Myers.*

WESTON, WILLIAM H. **A Petri dish holder for mechanical stages.** *Science*, 95, 415–6. 1942.

A Petri dish holder is described for use with mechanical stages, which consists essentially of a spring clip that firmly clasps the dish, and is carried in a metal frame that fits snugly into the slide holder of the movable stage. Readily available materials are used in construction and drawings showing the method of construction are included.—*T. M. McMillion.*

MICROTECHNIC IN GENERAL

LUBKIN, VIRGINIA, and CARSTEN, MARY. **Elimination of dehydration in histological technique.** *Science*, 95, 633–4. 1942.

A preliminary report is given on the successful use of RH–393 polyvinyl alcohol in 10% and 20% aqueous solution for infiltrating and embedding tissues, thereby eliminating the use of the usual dehydrating and clearing agents. The solution containing 20% by weight is prepared by suspending the powder in water at about 20° C., breaking up the lumps, then stirring well while heating to 75–85° C. Glycerin (20% by weight) is added to the solution as it cools. Washed tissues are placed directly in this solution, in shallow covered dishes, kept at room temperature except for a daily exposure of 2 hours to a temperature of 56° C. in the oven. Solidification takes place in 8 to 9 days. The trimmed block is attached to a fiber block by paraffin or cement, the cut sections unrolled on lukewarm water, and thereafter handled much like celloidin sections. Excellent staining is obtained with the usual stains and fat is retained in cells for staining with Sudan III.—*T. M. McMillion.*

OLSON, CARL, JR. **A device for marking fields on microscope slides.** *J. Lab. & Clin. Med.*, 27, 939–40. 1942.

A device for accurately marking a particular field on a slide can be prepared as follows: Knurl and thread one end of a brass cylinder so that it can be fitted into the nose-piece of a microscope. Reduce the other end to a cylindrical shaft $\frac{3}{16}$ in. in diameter and $\frac{3}{8}$ in. long. Slip a piece of rubber tubing over the shaft allowing it to project a short distance beyond its end. Cut the end of the rubber squarely across. Adjust the tubing so that its free end just touches the slide when the microscope is racked down. For operation, center the area to be marked in the field of the microscope. Ink the rubber tip lightly with the quill of an India ink bottle. Rack the body tube down until the end of the tube touches, leaving a ring of ink around the field to be marked. Removable tips with varying sized tubing may be prepared.—*John T. Myers.*

DYES AND THEIR BIOLOGICAL USES

BEYER, KARL H. **The color reactions of sympathomimetic amines with diazonium compounds.** *J. Amer. Chem. Soc.*, 64, 1318. 1942.

The coupling of p-nitrobenzenediazonium chloride with a sympathomimetic amine was first reported for the estimation of benzedrine (amphetamine). The adaptation of this color reaction to biological media made possible an investigation of the inactivation and excretion of that compound by man and dogs. Further study on the relation of structure of the diazonium compound and of the sympathomimetic amine to the color formed when these agents are coupled was necessary for the systematic identification as well as the colorimetric determination of these amines, as used in the example cited. The reaction characteristics of over thirty sympathomimetic amines with diazonium reagents are described. According to the spectrophotometric analyses of the colors formed as the result of these reactions, these compounds fall into three groups: Group 1, those having no hydroxyl group on the benzene ring but having a primary aliphatic amino group; group 2, those with coupling thru a monohydric phenol nucleus; group 3, those compounds having a catechol nucleus.—*A. P. Bradshaw.*

DEICHMANN, WILHELM, and SCHAFER, LAWRENCE J. **Spectrophotometric estimation of pentachlorophenol in tissues and water.** *Ind. Eng. Chem., Anal. Ed.*, 14, 310. 1942.

Pentachlorophenol, which is used in industry as an agent for the preservation of wood and wood products, has been found toxic in studies on mice. Extension of the investigation of the toxicity of this substance necessitated the development of an analytical method for the determination of minute amounts of pentachlorophenol in body tissues, blood and urine. The method suggested utilizes the spectrophotometric determination of a reddish-yellow pigment formed by the action of fuming HNO_3 on pentachlorophenol. The following is the method recommended: Distill 10 g. of macerated tissue with 20 ml. of water and 2 ml. of concentrated HCl until 1000 ml. of distillate have been collected in a flask containing 10 ml. of 4% NaOH. Concentrate half the distillate slowly to a final volume of 2 ml., and add gradually 4 ml. of fuming HNO_3. Loosen the precipitate formed with a stirring rod which is then rinsed with 1 ml. HNO_3. Place the solution in ice for 20 min., wash it with 100 ml. of water into a 250 ml. separatory funnel and extract the reddish-yellow pigment with three 8 ml. portions of chloroform (5 min. allowed for each separation). Wash the combined chloroform extracts with 100 ml. of water (10 min. allowed for the separation), then filter thru 2 layers of Whatman No. 1, 9 cm. filter paper wetted with chloroform into a 25 ml. volumetric flask. Bring the solution up to the mark with chloroform and determine the concentration by means of standard curves, using 10 mm. or 4-inch matched Aminco cells and the spectrophotometer at 460 mμ.—*A. P. Bradshaw.*

HECHTER, O., KROHN, L., and HARRIS, J. **Effects of estrogens and other steroids on capillary permeability.** *Endocrinology*, 30, 598. 1942.

The substances used were estradiol benzoate, estradiol, estrone, estriol, stilbestrol, desoxycorticosterone acetate, testosterone dipropionate and progesterone. Each of these steroid hormones increased the permeability of the capillaries of the uterus and vagina of rats, as shown by the uptake of trypan blue, but all were without significant effect on other tissues. The estrogens acted in much smaller doses (10 rat units) than the other hormones (1–5 mg.).—*L. Farber.*

KNAYSI, GEORGES. **Further studies on the use of basic dyes for measuring hydrolysis of fats.** *J. Dairy Sci.*, 25, 585–8. 1942.

The following dyes were used to determine the hydrolysis of fats: Nile blue A, C.I. No. 913, and spirit blue, Schultz No. 521, both from the National Aniline and Chemical Co., Inc.; Nile blue hydrochloride and methylene blue (rectified for blood stain), both from Coleman and Bell Co.; and Grübler neutral red. Dye bases were prepared from 0.2% solutions of the dye salts in water (or, in the case of spirit blue which is insoluble in water, in 75%, by volume, of ethyl alcohol)

by precipitation with N NaOH, of which 0.5–0.6 ml. was required for 50 ml. of solution. Following filtration, the precipitates were washed with water at pH 7.5 to 8.0. The precipitated base of Nile blu was impure, even tho the salt used was of relatively high purity. The other bases were relatively pure. All were stable in the dry form. The characteristics of solutions of the bases in water, 75% ethyl alcohol, xylol and buffer solutions are discussed for each dye. The Nile blue base was shown to be highly unstable. Neutral red seemed the most generally suitable of the three other dye bases studied, altho it had certain drawbacks for small globules and similar structures because of the somewhat poor contrast between the base color and that of its soaps. Use of dye bases for determining quality of edible fats has yielded promising preliminary results.—*F. E. Nelson.*

KNAYSI, GEORGES, and GUTHRIE, E. S. **A colorimetric method for estimating the quality of butter—a preliminary report.** *J. Dairy Sci.*, 25, 589–93. 1942.

The neutral red base used was made by precipitating 0.2 g. of neutral red dye in 50 ml. of water by the addition of 0.5 ml. of N NaOH, washing with water at pH 7.5–8.0 and drying. Butter oil was obtained by heating the butter sample to 60–70°C. for about 15 min. The test was made by mixing 1 ml. of butter oil with 3 ml. of saturated xylol solution of the base and comparing the resulting color with that of standards made by substituting oleic acid in known amounts dissolved in xylol for the butterfat of the test. The standards covered a range from zero to 30. It was sometimes necessary to remove turbidity by permitting the tests to stand for some hours or by centrifuging. The test was applied to more than a hundred samples of commercial or experimental butter. A sample which gave a test corresponding to a standard greater than 5 was considered of poor or bad quality and that corresponding to standard 10 was considered unfit for human consumption. Addition of salt or development of acidity by starter increased the hydrolysis of fat as shown by the test, but the effect was not due to the lactic and acetic acids in the starter. Addition of starter distillates or concentrates had no effect. The authors conclude that the test should have some value in assisting in the detection of poor butter.—*F. E. Nelson.*

TURNER, JOSEPH C., and MULLIKEN, BARBARA. **Production of subcutaneous sarcoma by azo dye and the influence thereon of liver feeding.** *Proc. Soc. Exp. Biol. & Med.*, 49, 317–9. 1942.

Repeated injections of p-dimethylaminoazobenzene (butter yellow) or o-aminoazotoluene in mice over a period of a year resulted in the development of sarcomata. The development of these tumors appeared not to be affected by the feeding of liver.—*M. S. Marshall.*

ANIMAL MICROTECHNIC

ANDERSON, J. **A rapid method for staining myelin in traumatic neuromas and injured nerves.** *J. Path. & Bact.*, 54, 258–9. 1942.

The following technic shortens the time of mordanting when frozen sections of nerves are to be stained by the Weigert-Pal method, and ". . has never failed to give good staining even of the finest myelin sheaths":

Freeze tissue hard and cut sections at 30 μ. Place for 1 hr., at 50°C. in the following: Weigert's primary mordant, 75 cc.; 4% aqueous phosphomolybdic acid, 16 cc.; 2% aqueous CaOCl (supernatant liquid), 8 cc. (Weigert's primary mordant consists of: $K_2Cr_2O_7$, 5 g.; CrF_3, 2.5 g.; water, 100 cc.)

Wash in water and stain in Kultschitsky's hematoxylin about 30 min. at 50°. Transfer to 2.5% $K_2Cr_2O_7$ for 2–3 min. Wash and differentiate by Pal's method. Counterstain in Anderson's alum-carmine for 45–60 min. at 50°C., or use Van Gieson's stain. Wash sections very rapidly in distilled water and transfer to 80% alcohol. Mount on slides, dehydrate in absolute alcohol, clear and mount in Canada balsam.—*S. H. Hutner.*

BLANK, HENRY S. **An improved routine tissue staining technic for formalin fixed tissues.** *J. Lab. & Clin. Med.*, 27, 1342–4. 1942.

Mercurochrome 220 can replace eosin in staining formalin-fixed paraffin sections, by the following technic: Deparaffinize the sections in 2 changes of xylol. Place in absolute alcohol, 1 min.; 95% alcohol, 1 min.; 70% alcohol, 1 min.; Delafield's hematoxylin, 5 min.; wash in 2 changes of water; decolorize until rose-colored in water containing a few drops of concentrated HCl; wash in water; wash until blue in water containing a few drops of strong NH_4OH; wash in water; place 1 min. in mercurochrome solution (2% mercurochrome 5 cc., alcohol 25 cc., water 70 cc.); wash in water; place 2 min. in a methylene-blue-azure solution prepared by the following method: dissolve 0.5 g. methylene blue in 10 cc. absolute alcohol and add 90 cc. 4% neutral formalin; dilute 4.5 cc. stock Giemsa stain to 100 cc. with 4% neutral formalin; mix the two solutions and add 1 g. borax to obtain the stock staining solution, which is diluted 1:20 for use. After staining, decolorize sections in 90% alcohol until no more stain comes off; complete the dehydration with 2 changes of absolute alcohol; clear in 2 changes of xylol; and mount in balsam. Mercurochrome does not diffuse as does eosin. The method is simple, giving excellent and uniform results.—*John T. Myers.*

GOODRICH, EDWIN S. **A new method of dissociating cells.** *Quart. J. Micr. Sci.*, 83, 245–58. 1942.

Small pieces of tissue, or small animals such as Hydra, are immersed in a saturated solution of boric acid in normal salt solution, to which has been added a trace (about 2 drops to 25 cc.) of Lugol's solution of iodine. At ordinary temperatures, several days are required to obtain a saturated solution of boric acid. After thoro impregnation, the specimen is placed in pure saturated boric acid solution in which it may remain for days or weeks. The method makes it possible to ascertain and to demonstrate the shapes of isolated cells of various animal tissues. It works best with epithelia having a free surface. In such cases the cells may fall off or may be pipetted off and placed on a slide. In other cases the cells must be separated by tearing with needles on the slide. By this method there is no solvent action on certain membranes, such as the connective tissue membrane which surrounds most organs of metazoa.

The dissociation may be combined with the use of a stain, such as hemalum, carmalum, toluidine blue, or methylene blue, which will mix with boric acid without precipitation. The stain is added to the boric acid solution, or a drop is introduced under the cover glass.—*C. E. Allen.*

MACK, H. C. **New and rapid method of staining vaginal smears based upon a specific reaction for glycogen.** *Harper Hosp. Bull.*, 1, 54. 1942.

1. Preparation of smears: A moistened cotton applicator is inserted into the vagina and twirled slightly (one complete rotation) against the vaginal wall. The cotton end of the applicator is then rolled lengthwise over the surface of a clean glass slide. By rolling, rather than rubbing, a uniformly thin film of cells, with minimal clumping and cell distortion, results. The film dries almost immediately and may be stained at once.

2. Staining of smears: Staining is accomplished simply by laying the slide, face down, over a shallow dish containing a small amount of Lugol's solution. Iodine vapors which arise insensibly from the solution suffice to stain the glycogen-containing cells in 2 or 3 min. Microscopic examination may be carried out immediately. Altho such stains fade in 24 to 48 hr., restaining (by the same method) may be carried out repeatedly if later examinations are desired.—(from *J. Lab. & Clin. Med.*, 27, 1104.)

PAPANICOLAOU, GEORGE N. **A new procedure for staining vaginal smears.** *Science*, 95, 438–9. 1942.

Two alcoholic solutions are described (denoted EA36 and EA25) which yield improved differentiation of cells in vaginal smears. In preparing the stains, stock solutions in 95% alcohol of 0.5% light green SF, 0.5% Bismarck brown, and 0.5% eosin yellowish are prepared, heat being used to dissolve the stains. National Aniline and Chemical Company dyes are used.

Stain EA 36 contains: 45 cc. light green SF; 10 cc. Bismarck brown; 45 cc. eosin, yellowish; 0.2 g. phosphotungstic acid; 1 drop saturated aqueous solution Li_2CO_3.

Stain EA 25 contains: 44 cc. 0.5% light green SF; 12 cc. Bismarck brown; 44 cc. eosin yellowish; 0.17 g. phosphotungstic acid; 1 drop saturated aqueous solution Li_2CO_3.

The following procedure is used:

Fix wet smears in equal parts ether and 95% alcohol, 5–15 min.; rinse successively in 70% alcohol, 50% alcohol, and distilled water; stain in hematoxylin 5–10 min.; rinse in distilled water; differentiate in 0.5% HCl; rinse in water, and leave 1 min. in dilute Li_2CO_3 (3 drops saturated aqueous solution in 100 cc. of water); rinse thoroly; rinse in 50%, 70%, 80%, 95% alcohols; stain 1 min. in 0.5% alcoholic orange G containing 0.015% phosphotungstic acid; rinse thoroly in 95% alcohol to remove all excess stain; stain 2 min. in EA 36 or EA 25; rinse 5 to 10 times in each of 3 jars of 95% alcohol; rinse in absolute alcohol and xylol. Mount in Clarite, Canada balsam, or gum damar. By this method, differentiation of cells is facilitated.—*T. M. McMillion.*

PERNOLKIS, EVANS W. **Simple method of evaluating blood platelets.** *J. Lab. and Clin. Med.*, 27, 1069. 1942.

The author proposes the following method as the simplest and least time-consuming for the identification and counting of blood platelets:

Place a drop of 1% brilliant cresyl blue in absolute alcohol on a clean slide, spread like a blood film and allow it to dry. Overlay with a small drop of blood. Make a red blood cell count at the same time. Count 1000 red cells and the platelets in the same area, which appear as pale blue bodies among the less colored red cells. From the red cell count, calculate the number of platelets per cu. mm.—*John T. Myers.*

SCHOOR, EPHRAIM. **A new technic for staining vaginal smears: III, A single differential stain.** *Science*, 94, 545. 1941.

A further modification of the Masson trichrome stain is described, in which a single differential staining solution is used to provide a sharp differentiation between cornified cells, stained orange red, and non-cornified cells, stained green. The stain (S3) is prepared by dissolving completely, in 100 cc. 50% alcohol, the following ingredients: 0.5 g. Biebrich scarlet W.S.; 0.25 g. orange G; 0.075 g. fast green FCF; 0.5 g. phosphotungstic acid C.P.; 0.5 g. phosphomolybdic acid C.P.; 1 cc. glacial acetic acid. National Aniline dyes are recommended.

The vaginal smears are fixed while wet for 1 to 2 minutes in equal parts of ether and 95% alcohol; stained for approximately 1 min. in solution S3; carried thru 70%, 95%, absolute alcohol, dipping the slide 10 times in each solution; cleared in xylol and mounted in damar. Variations of procedure in making smears and mounting are also discussed.—*T. M. McMillion.*

TAHMISIAN, THEODORE N., and SLIFER, ELEANOR H. **Sectioning and staining refractory materials in paraffin.** *Science*, 95, 284. 1942.

A new combination is outlined for using standard dioxan-paraffin procedures for sectioning some of the more difficult types of tissues (e.g., the vertebrate eye). The chief contribution is to suggest the following departures from the usual methods: to stain the sections, as soon as they are spread on water on a clean slide, with Mallory's triple connective tissue stain; and subsequently to cover them with a film of celloidin before clearing and mounting. Advantages claimed for the method over ordinary technics are that cellular details are well preserved, and normally hard tissues remain flat and do not curl away from the slide.—*T. M. McMillion.*

PLANT MICROTECHNIC

ASTBURY, W. T., and PRESTON, R. D. **The structure of the cell wall in some species of the filamentous green alga Cladophora.** *Proc. Roy. Soc. (B)*, 129, 54–76. 1940.

X-ray determinations of cell walls of three species of *Cladophora* were made on dried samples of filament which were treated with N/20 HCl to remove incrustations, washed in running water, and dried again. For microscopic observation specimens were fixed in 70% alcohol or in equal parts of 100% alcohol and a ⅛ saturated solution of picric acid in sea water. Material was left in this latter solution for 2–3 hr., washed in running water and immersed in a solution of NaOCl (1% available chlorine) for several hours. Material was ready immediately for observation. Critical determinations must be made within 24 hr. after treatment.—*T. E. Weier.*

MICROÖRGANISMS

FOURNELLE, H. J., and MACY, H. **A study of the coliform groups in ice cream.** *J. Dairy Sci.*, 25, 475–87. 1942.

Brilliant-green-lactose-bile 2% broth, the same broth buffered by the addition of 0.5% K_2HPO_4 (in the case of sherbets and ices) and violet-red-bile agar were used in testing for coliform bacteria in 69 factory-packed ice cream samples from fourteen manufacturers and in dipper samples from thirty retail stores. Brilliant green concentrations of 1:30,000 and 1:75,188 were tested in the broth and no significant difference could be found in the counts obtained. Buffering the broth medium was without significant effect, even tho addition of sherbet or ice to the unbuffered broth resulted in an appreciable drop in pH. Counts on violet-red-bile agar were essentially the same as those on brilliant green broth, but the latter medium was preferred because larger inocula could be used. *Escherichia freundii*, *Aerobacter aerogenes* and *Aerobacter cloacae* were the coliform organisms recovered most commonly.

Less than 10 coliform bacteria per ml. should be found in factory-packed ice cream samples.—*F. E. Nelson.*

KRAJIAN, A. A. **A new staining method for Gram-positive and Gram-negative organisms in frozen sections.** *Arch. Path.*, 32, 825. 1941.

Gram-positive and Gram-negative organisms in frozen sections may be distinguished by the following method: Prepare frozen sections, 7–10μ thick, in the usual manner. Stain 2 min. in alum-hematoxylin (Harris' method). Wash in tap water until blue, and destain rapidly in acid alcohol, dipping in and out 5 to 7 times. Rinse in tap water and apply 3 min. a copper-sulfate-zinc solution (7 g. $CuSO_4$ and 4 g. $ZnSO_4$ dissolved in 100 cc. distilled water with the aid of heat). Pour off, and apply for 5 min. a brilliant green solution (0.3 g. brilliant green dissolved in 10 cc. $CuSO_4$-$ZnSO_4$ mixture). Rinse in water and fortify for 1 min. with 5% aqueous NH_4NO_3. Rinse in tap water and apply carbol fuchsin (Ziehl-Neelsen method) for 2 min. Rinse in tap water, blot, and apply dioxan for 2 min. Pour off, and without washing, apply creosote-xylene (equal parts), changing the solution several times and agitating the slide for even differentiation until the background appears to be clear red with no more stain leaving the section. (This step requires about 1 min., and it is advisable to control the differentiation under the microscope.) Clear 2 min. in pure xylene. Mount in gum damar.

With the use of this method nuclei stain bluish-red; Gram-positive organisms, bluish green; Gram-negative organisms, red; monilias and actinomycetes, green; and Negri bodies, bright red with greenish chromatin bodies.

All the staining solutions are stable, except the brilliant green which keeps well for about 24 hr.—(from *J. Lab. & Clin. Med.*, 27, 977.)

LEE, JAMES SUMNER. **A combined fixative and stain for the cilia and trichocysts of Paramecium.** *Science*, 94, 352. 1941.

A new stain for cilia and trichocysts for which superior qualities are claimed is as follows: 50 cc. 5% $CuSO_4$; 12 drops 0.1*N* HCl; 5 drops blue ink. To stain the cilia only, omit the HCl from the formula. To use the stain, add two drops to the

culture on the slide, cover with cover glass and examine. The best preparations are usually found around the edges.—*T. M. McMillion.*

OLIVER, J., and REUSSER, T. R. **Rapid method for the concentration of tubercle bacilli.** *Amer. Rev. Tuberc.*, 45, 150. 1942.

The concentration of tubercle bacilli may be determined rapidly by the following method: Mix 5–10 ml. of sputum or other body fluid with an equal volume of Clorox (a bleach, deodorant and disinfectant manufactured by the Clorox Chemical Company); shake two or three times over a two-minute period and let stand at room temperature for 10 min.; centrifuge the mixture at 3000 r.p.m. for 10 min. in a 15 ml. conical tube; pour off the supernatant fluid and drain for 2 min.; transfer a drop of the creamy-white sediment to a slide and stain as desired. The smear dries very quickly and needs no fixing.—*L. Farber.*

POTTENGER, J. E. **Controlled staining of Mycobacterium tuberculosis.** *Amer. Rev. Tuberc.*, 45, 548. 1942.

To check the staining procedure for the tubercle bacillus in unknown specimens the author recommends placing a smear of a known suspension of the organisms on the same slide as the unknown. The degree of staining of the known smear is an index of the staining technic. The details are as follows:

Preparation of control: Shake a mucoid specimen of sputum, containing one to three organisms per field, with glass beads to distribute the bacilli uniformly, and store at 5°C.; prepare a smear varying in thickness from very thin to overthick.

Preparation of suspected material: Dilute from 3 to 10 times with saline and homogenize by mechanical shaking for 10 min. Prepare a smear about 2 cm. × 0.5 cm. and varying in thickness from very thin to overthick. In order to avoid spattering of the unknown smear by the control during drying, place a sterile glass slide, inserted vertically into a groove cut in the side of a cork, between the two smears.

Staining procedure: Stain 15 min. on top of a closed water bath at 65°C. in a mixture of 16 g. basic fuchsin, 150 ml. 99% alcohol, and 50 ml. phenol, made up to a liter with water; cool 2–3 min.; apply 3% HCl, 30 sec.; wash 10 sec. with 70% alcohol or completely decolorize with freshly prepared 5% Na_2SO_3 and 70% alcohol alternately; wash with water; counterstain with ⅓ saturated aqueous picric acid, 10 sec. The extent of staining is classified as deep, moderate, and pale. The author recommends that at least 70% of all the organisms observed in the control be deeply stained and that not over 10% be pale. If this differential is not observed in the control smear, the slide should be restained. The hazard of contaminating the unknown by the control smear is discussed and is concluded to be negligible.—*L. Farber.*

POTTENGER, J. E. **Counterstains in demonstrating Mycobacterium tuberculosis in sputum.** *Amer. Rev. Tuberc.*, 45, 558. 1942.

This report deals with the effect of various counterstains on the visibility of tubercle bacilli in stained smears. Carefully prepared smears were treated as follows: Stain 1 min. in carbol fuchsin at 65°C.; pour off stain and flood with 5% H_2SO_4, 30 sec.; immerse in a beaker of water until no more stain is removed; completely decolorize by Osol's method (Deut. med. Woch., No. 24, 1008, 1907), using a 5:1 mixture of 10% Na_2SO_3 and 96% alcohol. The following counterstains were then applied: 1% methylene blue for 30 sec.; 0.1% methylene blue for 30 sec.; 0.0067% methylene blue for 1 min.; 1% Bismarck brown for 2 min.; 0.05% Bismarck brown for 1 min.; ⅓ saturated malachite green for 1 min.; 0.02% malachite green for 1 min.; 1% picric acid for 5–10 sec. A 10-minute count of organisms for each counterstain was made. The relative efficiencies of the counterstains, based upon the sum of the highest counts for each of six sputa, were as follows: 1% methylene blue—1.0; 0.1% methylene blue—1.6; 1% Bismarck brown—2.2; ⅓ saturated malachite green—2.6; 0.05% Bismarck brown—3.7; 0.02% malachite green—3.9; 0.0067% methylene blue—4.5; no counterstain—4.8; 1% picric acid—6.2. The superiority of picric acid as a counterstain was more clearly observed using smears containing few organisms. The author recommends the use of picric acid and similar light counterstains in preference to dark

ones which should be dispensed with in order to obtain maximum efficiency from the staining procedure.—*L. Farber.*

SISTER MARY JOHN. **Sulphonamides and the Ziehl-Neelsen stain.** *Amer. J. Med. Techn.*, **7**, 256. 1941.

Observations are reported showing that after the administration of sulfonamide drugs the sputum may contain crystals which are acid-fast and which may rather closely resemble the tubercle organism.

These are seen as long slender, acid-fast rods, sometimes curved, sometimes short and straight, sometimes in bristling clumps.

It is advisable that these drugs be discontinued before sputum examinations for tubercle bacilli are reported as positive.—(from *J. Lab. & Clin. Med.*, 27, 1106.)

VERHOEFF, F. H. **Improved method of staining within tissues, leptothriches of Parinaud's conjunctivitis and Gram positive organisms.** *J. Amer. Med. Assn.*, **115**, 1546. 1940.

Staining of leptothriches located in the tissues by the ordinary Gram method is not wholly satisfactory, because, if alcohol or anilin alone is used, the organisms as well as the tissues are decolorized. The author has previously described a method of staining (*Arch. Ophth.*, **42**, 345, 1913) in which chloroform was employed as one of the solvents and the sections transferred alternately from alcohol to oil to decolorize tissues but check decolorization of the organisms. A series of tests has resulted in the substitution of trichloro-ethylene for chloroform, because it is less apt to decolorize organisms in tissues, forms fewer precipitates and is equally satisfactory for fixation with Zenker's solution as with formaldehyde.

The details of this new modification of the Gram stain for leptothriches are as follows: Fix fresh tissues in Zenker's solution or formaldehyde. Embed in celloidin or paraffin and cut thin sections. Stain in hematoxylin and eosin. Examine after mounting and select sections containing largest foci of macrophages. Remove sections from slides with xylene. Wash successively in oil of thyme, in 95% alcohol, and in water. Immerse celloidin sections in solvents. (Use droppers in case of paraffin sections.) Wash in water. Place section on a slide (remove excess water) and stain 2 min. with Stirling's crystal violet, (5 g. in 10 ml. 95% ethyl alcohol; anilin, 2 cc.; distilled water, 88 cc.). Wash in several changes of water for 2 min. Immerse 20–60 sec. in compound solution of iodine (iodine, 1 g.; KI, 2 g.; water, 100 cc.). Wash in water and agitate 5 sec. in 95% alcohol until stain comes out freely, then transfer quickly to oil of thyme for 1 min. Transfer to 95% alcohol for 5 sec., and again to oil of thyme. Examine under low power. If necessary, repeat differentiation. Wash in 2 changes of xylene, mount in cedar oil on slide.

Leptothriches should be deeply stained and easily recognized when in masses; single organisms are more difficult to detect. This method is superior to other Gram-stain modifications for staining Gram-positive organisms within tissues and even Gram-negative meningococci have been successfully stained by this method.—*Elizabeth F. Genung.*

WHEELER, S. M., and FOLEY, G. E. **Pigment observed in cultures of hemolytic streptococci belonging to Lancefield Group A.** *Proc. Soc. Exp. Biol. & Med.*, **49**, 421–4. 1942.

A yellowish-brown pigment was recovered from several of a series of Group A hemolytic streptococci. The pigment was confined to the colony or to the sediment in broth cultures. It was soluble in ether, chloroform, carbon disulfide, and to some extent, in alcohol. It seemed to be related to the carotinoids. Spectrophotometrically, a light between 4400 and 5400 A° was best transmitted. Pigment production seemed to be caused by environment rather than to be a group or type characteristic.—*M. S. Marshall.*

INDEX TO VOLUME 17

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since those listed in the last number of this Journal.

STAINS CERTIFIED JUNE 1, TO AUG. 31, 1942*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Giemsa stain	GGe–9		Blood staining	June 5, 1942
Crystal violet	CC–14	88%	Histology; cytology; bacteriology (staining; in media)	June 6, 1942
Fast green FCF	LGf–2	93%	Histology; cytology	June 15, 1942
Safranin O	CS–10	86%	Histology; cytology; bacteriology (staining)	June 17, 1942
Giemsa stain	CGe–2		Blood staining	June 29, 1942
Erythrosin B	CEr–4	79%	Histology; bacteriology (staining)	June 30, 1942
Eosin Y	CE–14	85%	Histology; compounding of blood stains	June 30, 1942
Orcein	FOr–1		Animal histology	July 2, 1942
Fast green FCF	CGf–2	88%	Histology; cytology	July 2, 1942
Sudan III	CY–6	77%	Fat staining	July 2, 1942
Safranin O	NS–16	85%	Histology; cytology; bacteriology (staining)	July 3, 1942
Fuchsin, basic	LF–9	95%	Histology; Feulgen reaction; bacteriology (staining; in media)	July 7, 1942
Azocarmine G	NAg–1	77%	Histology	July 13, 1942
Methylene blue thiocyanate tablets	NAt–3	1.59–1.67%	Testing reduction in milk	July 16, 1942
Hematoxylin	LH–1	...	Histology; cytology	July 16, 1942
Brilliant cresyl blue	CV–2	47%	Vital blood staining	July 17, 1942
Methylene blue chloride	NA–21	84%	Histology; bacteriology (staining) compounding of blood stains	July 20, 1942
Fuchsin, acid	NR–13	50%	Histology; Andrade indicator	July 23, 1942
Giemsa stain	NGe–12		Blood staining	July 24, 1942
Fuchsin, basic	NF–38	87%	Histology; Feulgen reaction; bacteriology (staining; in media)	July 29, 1942
Fuchsin, basic	LF–10	93%	Histology; Feulgen reaction; bacteriology (staining; in media)	Aug. 3, 1942

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAINS CERTIFIED JUNE 1, TO AUG. 31, 1942* *Concluded*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Crystal violet	NC–2	94%	Histology; cytology; bacteriology (staining; in media)	Aug. 10, 1942
Azure C	NAc–3		Histology	Aug. 12, 1942
Congo red	NQ–11	89%	Histology	Aug. 13, 1942
Fuchsin, basic	NF–39	93%	Histology; Feulgen reaction; bacteriology (staining; in media)	Aug. 13, 1942
Nigrosin	NNi–8		Bacteriology (staining)	Aug. 28, 1942
Malachite green	NMg–10		Histology; bacteriology (staining)	Aug. 28, 1942
Bismarck brown Y	CN–6	51%	Histology; bacteriology (staining)	Aug. 28, 1942
Fuchsin, acid	CR–9	73%	Histology; Andrade indicator	Aug. 28, 1942

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check to its staining value. Certification does not in any instance, however, imply approval for medicinal use.